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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 16/00, C07H 21/04, C12P 21/06, A61K 38/00

A1

(11) International Publication Number: WO 99/26980

(43) International Publication Date: 3 June 1999 (03.06.99)

(21) International Application Number: PCT/US98/24976

(22) International Filing Date: 19 November 1998 (19.11.98)

(30) Priority Data:

08/976,096	21 November 1997 (21.11.97)	US
60/099,160	4 September 1998 (04.09.98)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	08/976,096 (CIP)
Filed on	21 November 1997 (21.11.97)
US	60/099,160 (CIP)
Filed on	4 September 1998 (04.09.98)

(71) Applicant *(for all designated States except US)*: BETH ISRAEL DEACONESS MEDICAL CENTER [US/US]; 330 Brookline Avenue, Boston, MA 02215 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: TERHORST, Cornelis, P. [US/US]; 72 Fayerweather Street, Cambridge, MA 02215 (US). SAYOS–ORTEGA, Joan [ES/US]; 143 Park Drive #12, Boston, MA 02110–2214 (US).

(74) Agent: BIEKER–BRADY, Kristina; Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110–2214 (US).

(81) Designated States: AU, CA, JP, NZ, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHODS AND REAGENTS FOR THE UTILIZATION OF SAP FAMILY MEMBER PROTEINS, NOVEL SIGNAL TRANSDUCTION REGULATORS

(57) Abstract

The invention provides novel SAP proteins and nucleic acid sequences. The invention also identifies the SAP family, members of which are novel signal transduction regulators. Also provided are SAP family polypeptides, SAP family member polypeptide specific antibodies, and methods for modulating SH2 domain–containing protein–mediated signal transduction, in particular, antigen–specific T cell activation. In addition, methods are provided for detecting compounds for modulating SH2 domain–containing protein–mediated signal transduction.

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METHODS AND REAGENTS FOR THE UTILIZATION OF SAP FAMILY MEMBER PROTEINS, NOVEL SIGNAL TRANSDUCTION REGULATORS

Background of the Invention

The invention relates to modulation of SH2 domain-containing protein-mediated signal transduction generally, and in particular antigen-specific T cell activation.

The signal transduction cascade emanating from a cell surface-localized receptor into the nucleus of the cell where activation-specific genes are upregulated involves a very large number of molecules, including kinases, phosphatases, and adaptor molecules. Interestingly, the molecules involved in signal transduction events in both B and T lymphocytes, as well as in other non-lymphoid cells, both hematopoietic and non-hematopoietic, often bear an internal domain with homology to a domain in the *src* p60 tyrosine kinase found in platelets and neuronal tissues. This domain, termed an SH2 domain, has been found to bind to phosphorylated tyrosine residues, thus mediating the interaction of molecules involved in the signal transduction cascade in a variety of cells.

The Signaling Lymphocyte Activation Molecule (SLAM) (CDw150), a 70kD glycosylated type I transmembrane protein present on the surface of B and T cells, is a high-affinity self-ligand. Since triggering of SLAM co-activates T or B lymphocyte responses, it is considered to play a major role in bi-directional T↔B cell stimulation (Cocks *et al.*, Nature 376: 260-263, 1995; Aversa *et al.*, Immunol. Cell Biol. 75: 202-205, 1997; Aversa *et al.*, J. Immunol. 158: 4036-4044, 1997; Carballido *et al.*, J. Immunol. 159: 4316-4321, 1997; Isomaki *et al.*, J. Immunol. 159: 2986-2993, 1997; Punnonen *et al.*, J. Exp. Med. 185: 993-1004, 1997; Ferrante *et al.*, J. Immunol. 160: 1514-1521, 1998). Moreover, triggering by anti-SLAM antibodies induces the interferon-γ gene and redirects Th2 responses of antigen-specific T cell clones to a Th1 or Th0 phenotype (Aversa *et al.*, Immunol. Cell Biol. 75: 202-205, 1997; Aversa

et al., J. Immunol. 158: 4036-4044, 1997; Carballido *et al.*, *supra*). Furthermore, since anti-SLAM antibodies alone fail to enhance B cell proliferation, and produce, if anything, an inhibitory effect (Punnonen *et al.*, *supra*), SLAM induced signal transduction events in T lymphocytes would appear to be different from those in B cells.

Given the important role of SH2 domain-containing protein mediated signal transduction pathways, in particular the pathways involved in the immune system, novel methods and reagents for modulating such pathways are useful in treating a myriad of human conditions involving inappropriate levels of such SH2 domain-containing protein signal transduction.

Summary of the Invention

Described herein is a novel T cell specific-protein, SAP (SLAM-Associated Protein), that contains an SH2-domain and a short tail. Additionally disclosed is the identification of a novel family of signal transduction regulators, namely the SAP family. SAP family member proteins block the recruitment of SH2-domain containing signal transduction molecules to a docking site in the SLAM cytoplasmic domain.

We mapped the SAP protein-encoding gene to the locus for X-linked proliferative disease (XLP), and mutations in the SAP gene were found in three XLP patients. Our findings led us to the conclusion that the absence of SAP in XLP patients affects T / B cell interactions induced by SLAM, which leads to an inability to control B cell proliferation induced by EBV infections. Our results demonstrate that the SAP protein plays a key role in immunoregulation, and that SAP family member proteins play an important role in SH2 domain-containing protein mediated signal transduction.

According, in a first aspect, the invention provides a substantially pure nucleic acid encoding a SAP polypeptide. In one embodiment, the SAP polypeptide is a fragment of the full length naturally-occurring SAP polypeptide. In another

embodiment, the nucleic acid is operably linked to a second nucleic acid. The second nucleic acid sequence may be a coding sequence and the nucleic acid operably linked to said second nucleic acid produces a fusion protein that includes the SAP polypeptide. The second nucleic acid sequence may also be a gene promoter. In another embodiment, nucleic acid comprises a mutation that results in an amino acid alteration in the SAP polypeptide. In preferred embodiments of this aspect of the invention, the nucleic acid includes a nucleic acid sequence that is substantially identical to SEQ ID NO: 3 or SEQ ID NO: 5. In other embodiments, the SAP polypeptide binds a phosphorylated tyrosine residue, or binds a non-phosphorylated tyrosine residue. In yet another embodiment, the nucleic acid includes a nucleic acid sequence encoding a naturally-occurring SH2 domain.

In another embodiment of the first aspect of the invention, the nucleic acid encodes a SAP polypeptide that has SAP biological activity. In other embodiments, the SAP polypeptide modulates SH2 domain-containing protein-mediated signal transduction, such as antigen-specific T cell activation, which may be mediated by Th1 cells.

In a second aspect, the invention provides a substantially pure SAP polypeptide. In one embodiment, the polypeptide is a fragment of the full length naturally-occurring SAP polypeptide. In another embodiment, the polypeptide is part of a fusion protein. In yet another embodiment, the polypeptide includes an amino acid sequence that has a mutation as compared to the naturally-occurring amino acid sequence of the polypeptide. In preferred embodiments of this aspect of the invention, the SAP polypeptide includes an amino acid sequence that is substantially identical to SEQ ID NO: 4 or SEQ ID NO: 6. In other embodiments, the SAP polypeptide binds a phosphorylated tyrosine residue, or binds a non-phosphorylated tyrosine residue. In yet another embodiment, the polypeptide includes a naturally-occurring SH2 domain.

In another embodiment of the second aspect of the invention, the SAP polypeptide has SAP biological activity. In other embodiments, the polypeptide

modulates SH2 domain-containing protein-mediated signal transduction, such as antigen-specific T cell activation which may be mediated by Th1 cells.

In a third aspect, the invention provides a method for detecting a disease involving aberrant SH2 domain-containing protein-mediated signal transduction in a patient that includes: (a) isolating a cell from the patient and (b) measuring the level of expression of a SAP family member polypeptide in the cell, where an alteration in the level in the patient relative to the level in a cell from a healthy control indicates the presence of a disease involving aberrant SH2 domain-containing protein-mediated signal transduction in the patient. Preferably, the disease is X-linked proliferative disease.

In a fourth aspect, the invention provides a method for detecting a disease involving aberrant SH2 domain-containing protein-mediated signal transduction in a patient that includes: (a) isolating a SAP family member polypeptide from the patient and (b) determining the amino acid sequence of the SAP family member polypeptide, where an alteration in the amino acid sequence in the patient relative to the amino acid sequence of a SAP family member polypeptide isolated from a healthy control indicates the presence of a disease involving aberrant SH2 domain-containing protein-mediated signal transduction in the patient. Preferably, the disease is X-linked proliferative disease.

In a fifth aspect, the invention provides a method for treating a disease involving aberrant SH2 domain-containing protein-mediated signal transduction in a patient that includes administering to the patient a SAP family member polypeptide or a fragment, mutant, or fusion thereof.

In various embodiments of the third, fourth, and fifth aspects of the invention, the SAP family member polypeptide includes an amino acid sequence substantially identical to SEQ ID NO: 4 or SEQ ID NO: 6. Preferably, the disease is X-linked proliferative disease. In other embodiments, the SAP family member polypeptide is EAT-2. In other embodiments, the SAP polypeptide binds a phosphorylated tyrosine residue or non-phosphorylated tyrosine residue. In another embodiment, the SH2

domain-containing protein-mediated signal transduction is antigen-specific T cell activation, which may be mediated by Th1 cells.

In a sixth aspect, the invention provides a method for identifying a compound that modulates SH2 domain-containing protein-mediated signal transduction that includes: (a) providing a cell that includes a SAP family member-encoding gene; (b) contacting the cell with a candidate compound; and (c) monitoring expression of the SAP family member-encoding gene, where an alteration in the level of the expression of the gene in response to the candidate compound indicates the presence of a compound that modulates SH2 domain-containing protein-mediated signal transduction. In one embodiment of this aspect, the SAP family member-encoding gene encodes a SAP polypeptide. In another embodiment, the SAP family member encoding gene encodes an EAT-2 polypeptide.

In a seventh aspect, the invention provides a method for identifying a compound that modulates SH2 domain-containing protein-mediated signal transduction that includes: (a) providing a cell including a reporter gene operably linked to a promoter from a SAP family member encoding gene; (b) contacting the cell with a candidate compound; and (c) measuring expression of the reporter gene, where an alteration in the level of the expression of the reporter gene in response to the candidate compound indicates the presence of a compound that modulates SH2 domain-containing protein-mediated signal transduction. In one embodiment of this aspect, the SAP family member-encoding gene encodes a SAP polypeptide. In another embodiment, the SAP family member encoding gene encodes an EAT-2 polypeptide.

In an eighth aspect, the invention provides a method for identifying a compound that modulates SH2 domain-containing protein-mediated signal transduction that includes: (a) providing a cell having: (i) a reporter gene operably linked to a DNA-binding-protein recognition site; (ii) a first fusion gene capable of expressing a first fusion protein that includes a SAP family member polypeptide covalently bonded to a binding moiety that is capable of specifically binding to the

DNA-binding-protein recognition site; and (iii) a second fusion gene capable of expressing a second fusion protein that includes a SLAM polypeptide covalently bonded to a gene activating moiety; (b) exposing the cell to a candidate compound; and (c) measuring reporter gene expression in the cell, where an alteration in the level of the expression of the reporter gene in response to the candidate compound indicates the presence of a compound that modulates SH2 domain-containing protein-mediated signal transduction. In one embodiment of the eighth aspect, the cell is a yeast cell.

In a ninth aspect, the invention provides a method for identifying a compound that modulates SH2 domain-containing protein-mediated signal transduction that includes: (a) providing a cell having: (i) a reporter gene operably linked to a DNA-binding-protein recognition site; (ii) a first fusion gene capable of expressing a first fusion protein that includes a SLAM polypeptide covalently bonded to a binding moiety that is capable of specifically binding to the DNA-binding-protein recognition site; and (iii) a second fusion gene capable of expressing a second fusion protein that includes a SAP family member polypeptide covalently bonded to a gene activating moiety; (b) exposing the cell to the compound; and (c) measuring reporter gene expression in the cell, where an alteration in the level of the expression of the reporter gene in response to the candidate compound indicates the presence of a compound that modulates SH2 domain-containing protein-mediated signal transduction. In one embodiment of this aspect of the invention, the cell is a yeast cell.

In various embodiments of the sixth, seventh, eighth, and ninth aspects of the invention, the SH2 domain-containing protein-mediated signal transduction is antigen-specific T cell activation, which may be mediated by Th1 cells, and where the alteration is an increase indicates the compound increases antigen-specific T cell activation, and the alteration is a decrease indicates the compound decreases antigen-specific T cell activation. In other preferred embodiments, the SAP family member polypeptide is substantially identical to SEQ ID NO: 4 or SEQ ID NO: 6. In other embodiments, the SAP family member polypeptide is EAT-2.

In a tenth aspect, the invention provides a method for identifying a compound

as a SAP family member mimetic that includes the steps of: (a) providing a SLAM polypeptide, or a fragment or fusion thereof bearing non-phosphorylated tyrosine residues; (b) contacting the SLAM polypeptide with a SAP family member polypeptide; (c) contacting the SLAM polypeptide and the SAP family member polypeptide with a candidate compound; and (d) measuring the level of interaction of the SLAM polypeptide with the SAP family member polypeptide, where a decrease in the level in response to the compound relative to a level not contacted with the compound indicates that the compound is a SAP family member mimetic. In one embodiment, the SLAM polypeptide is bound to a solid state substrate.

In an eleventh aspect, the invention provides a method for identifying a compound as a SAP family member mimetic that includes the steps of: (a) providing a SLAM polypeptide, or a fragment or fusion thereof, the SLAM polypeptide bearing non-phosphorylated tyrosine residues; and (b) contacting the SLAM polypeptide with a candidate compound, where the candidate compound binding to the SLAM polypeptide indicates that the candidate compound is a SAP family member mimetic. In one embodiment of this aspect of the invention, the SLAM polypeptide is bound to a solid state substrate.

In a twelfth aspect, the invention features a method for identifying a polypeptide that modulates SH2 domain-containing protein-mediated signal transduction that includes: (a) providing a cell having: (i) a reporter gene operably linked to a DNA-binding-protein recognition site; (ii) a first fusion gene capable of expressing a first fusion protein that includes a SAP family member polypeptide covalently bonded to a binding moiety that is capable of specifically binding to the DNA-binding-protein recognition site; and (iii) a second fusion gene capable of expressing a second fusion protein that is selected from a library that includes a polypeptide covalently bonded to a gene activating moiety that is encoded by a cDNA of the library; and (b) measuring reporter gene expression in the cell, where an increase in the reporter gene expression identifies the presence of a polypeptide that modulates SH2 domain-containing protein-mediated signal transduction. In a

preferred embodiment, the cell is a yeast cell. In another embodiment of this aspect of the invention, the SH2 domain-containing protein-mediated signal transduction is antigen-specific T cell activation, which may be mediated by Th1 cells. In yet another embodiment, the library is constructed from a cell selected from a group consisting of a T cell, a B cell, a myeloid cell, a natural killer cell, a hepatocyte, a liver cell, a thymocyte, a hematopoietic progenitor cell, a fibroblast, a muscle cell, and a neuron.

In a thirteenth aspect, the invention provides a method for modulating SH2 domain-containing protein-mediated signal transduction in a mammal that includes providing a transgene encoding a SAP family member polypeptide or fragment thereof to a cell of the mammal, where the transgene is positioned for expression in the cell. In a preferred embodiment of this aspect of the invention, the mammal is selected from a group consisting of a rodent (*e.g.*, a mouse), a primate (*e.g.*, a chimpanzee), a ruminant (*e.g.*, a cow), a pig, a horse, a sheep, and a goat.

In a fourteenth aspect, the invention provides a method for modulating SH2 domain-containing protein-mediated signal transduction in a mammal that includes administering to a cell of the mammal a compound which modulates SAP family member biological activity. In a preferred embodiment of this aspect of the invention, the mammal is selected from a group consisting of a rodent (*e.g.*, a mouse), a primate (*e.g.*, a chimpanzee), a ruminant (*e.g.*, a cow), a pig, a horse, a sheep, and a goat.

In a fifteenth aspect, the invention provides a method for increasing antigen-specific T cell activation in a mammal that includes providing a transgene encoding a SAP family member polypeptide, the transgene being positioned for expression in the cell. In a preferred embodiment of this aspect of the invention, the mammal is selected from a group consisting of a rodent (*e.g.*, a mouse), a primate (*e.g.*, a chimpanzee), a ruminant (*e.g.*, a cow), a pig, a horse, a sheep, and a goat. Preferably, the antigen-specific T cell activation is mediated by Th1 helper T cells.

In a sixteenth aspect, the invention provides a method for increasing antigen-specific T cell activation in a mammal that includes administering to a cell of the

mammal a compound which increases the biological activity of a SAP family member protein. In a preferred embodiment of this aspect of the invention, the mammal is selected from a group consisting of a rodent (*e.g.*, a mouse), a primate (*e.g.*, a chimpanzee), a ruminant (*e.g.*, a cow), a pig, a horse, a sheep, and a goat. Preferably, the antigen-specific T cell activation is mediated by Th1 helper T cells.

In a seventeenth aspect, the invention features a transgenic mammal having a knockout mutation in an endogenous SAP family member protein-encoding nucleic acid sequence. Preferably, the SAP family member protein is a SAP protein or is an EAT-2 protein. In a preferred embodiment of this aspect of the invention, the mammal has altered SH2 domain-containing protein signal transduction relative to a mammal lacking an alteration in wild-type SAP family member-encoding nucleic acid sequences. In another preferred embodiment of this aspect of the invention, the mammal is selected from a group consisting of a rodent (*e.g.*, a mouse), a primate (*e.g.*, a chimpanzee), a ruminant (*e.g.*, a cow), a pig, a horse, a sheep, and a goat.

In an eighteenth aspect, the invention features a transgenic mammal having an exogenous SAP family member protein-encoding nucleic acid sequence operably linked to a promoter. Preferably, the SAP family member protein is a SAP protein or is an EAT-2 protein. In a preferred embodiment of this aspect of the invention, the mammal has altered SH2 domain-containing protein signal transduction relative to a mammal lacking an exogenous SAP family member-encoding nucleic acid sequence operably linked to a promoter. In another preferred embodiment of this aspect of the invention, the mammal is selected from a group consisting of a rodent (*e.g.*, a mouse), a primate (*e.g.*, a chimpanzee), a ruminant (*e.g.*, a cow), a pig, a horse, a sheep, and a goat.

In accordance with the present invention, by purified "SAP family member," or "SAP family member polypeptide" is meant an isolated SH2 domain with SAP biological activity, particularly the ability to bind either a phosphorylated or a non-phosphorylated tyrosine residue. A SAP family member polypeptide has fewer than

40 amino acids, preferably fewer than 35 amino acids, located N-terminally or C-terminally to the SH2 domain in the native polypeptide. Preferably, the non-SH2 domain amino acids have no enzymatic activity. For example, if a fusion protein is made using, as one of the fusion partners an isolated SH2 domain excised from an SH2 domain-containing protein, the fusion protein is a SAP family member polypeptide if not more than 40 amino acids from the N-terminally and/or C-terminally located amino acids immediately adjacent to the SH2 domain in the native SH2 domain-containing protein are incorporated into the fusion protein. A preferred SAP family member polypeptide modulates signal transduction pathways involving SH2 domain-containing proteins. Exemplary SAP family member proteins are the SAP proteins and the EAT-2 proteins described herein.

By purified "SAP," "SAP protein," or "SAP polypeptide" is meant a protein or polypeptide having at least 70%, preferably at least 80%, more preferably at least 90%, and most preferably at least 95% overall identity with the amino acid sequence of human SAP (SEQ ID NO: 4) or murine SAP (SEQ ID NO: 6), as shown in Figs. 2B and 2D, respectively. Polypeptide products from splice variants of SAP gene sequences are also included in this definition. Preferably, the SAP protein is encoded by nucleic acid having a sequence with hybridizes to the nucleic acid sequence of Figs. 2A or 2C under high stringency conditions. Proteins and polypeptides localized anywhere in a cell are included in the definition. For example, a SAP polypeptide may be nucleus-localized, or membrane attached through myristoylation or palmitoylation addition.

By "SAP family member specific antibody" is meant an antibody (*i.e.*, monoclonal or polyclonal) that binds a SAP family member polypeptide, such as the human or mouse SAP proteins or EAT-2 proteins described herein. Specifically excluded from the definition is an antibody that binds an SH2 domain-containing protein, as defined below. Preferably, the SAP family member specific antibody of the invention is a rabbit polyclonal antibody-containing antisera described below.

By "SH2 domain" is meant a polypeptide domain which is defined by the

presence of "blocks" found in many proteins initially identified as having sequence identity to a domain in src tyrosine kinase that defines the second src homology (SH2) domain. Current practice in the field has determined the presence of an SH2 domain based on the close identity of "blocks" of sequences within the putative SH2 domain with "blocks" found in other SH2-domain containing proteins. Such analysis can be made using the position-based method of Henikoff and Henikoff, J. Mol. Biol. 243: 574-578, 1994; Henikoff and Henikoff, Genomics 19: 97-107, 1994. For example, shown in Fig. 4A is the SAP amino acid sequence with the positions of the five SH2 domain-hallmarking "blocks" shown in bold. In Fig. 4B, the sequences of the blocks in human SAP is shown. Each of these "blocks" has a high degree (*e.g.*, above 60%) sequence identity with a corresponding block in the SH2 domain of another SH2 domain-containing protein. Although as a entire family, the SH2 domain of one protein may show a moderate degree of identity with a second SH2 domain-containing protein; however the sequence identity across "blocks" identify an SH2 domain-containing protein.

By "SH2 domain-containing protein" or "SH2 domain-containing polypeptide" is meant a protein or polypeptide which has an SH2 domain, as well as at least 50 amino acid residues located N-terminal or C-terminal to the SH2 domain. Hence, specifically excluded from the definition of SH2 domain-containing polypeptides are SAP family member polypeptides. Preferably, the SH2 domain of an SH2-domain containing protein binds phosphorylated tyrosine residues, but does not bind non-phosphorylated tyrosine residues. The amino acids located N-terminal or C-terminal to the SH2 domain of an SH2 domain-containing protein may have functional activity (*e.g.*, another SH2 domain, an SH3 domain, a kinase domain, or a phosphatase domain).

By "SLAM" is meant the protein or polypeptide which is an isoform (*i.e.*, a gene splice variant) of SLAM, a multifunctional 70 kDa glycoprotein member of the Ig superfamily. SLAM is a high affinity self-ligand and is characterized by its rapid induction on naive T cells and B cells following activation of these cells. The four

SLAM isoforms currently known are SLAM 1 (or simply SLAM, the 70 kDa glycoprotein), SLAM2, SLAM3, and SLAM4.

By "SAP biological activity" or "SAP family member biological activity" is meant any one or more of the biological activities described herein for any of the SAP family member polypeptides described herein, including, without limitation, the ability to bind to a non-phosphoryated tyrosine residue, the ability to bind to a phosphoryated tyrosine residue, and the ability to counteract signaling activity of an SH2 domain-containing polypeptide. The non-phosphorylated tyrosine residue is preferably present in any polypeptide. Most preferably, the non-phosphorylated tyrosine residue is present in the cytoplasmic domain of the SLAM polypeptide.

By "SH2 domain-containing protein-mediated signal transduction" is meant a signal transduction event or signal transduction pathway in which an SH2 domain-containing protein plays a role. For example, the platelet derived growth factor (PDGF) receptor-mediated signal transduction pathway is an SH2 domain-containing protein-mediated signal transduction because the pathway involves at least one SH2 domain-containing protein.

By "modulating SH2 domain-containing protein-mediated signal transduction" is meant increasing (*i.e.*, enhancing) or decreasing (*i.e.*, inhibiting) the intracellular signaling involving SH2 domain-containing proteins in a given cell population relative to a control cell population not exposed to a test compound. Preferably, the increase or decrease in the given cell population exposed to a test compound is a change of at least 25%, more preferably the change is at least 50%, and most preferably the change is at least one-fold, as compared to a control cell population. SH2 domain-containing protein-mediated signal transduction may be measured by a variety of assays known in the art, including the assays described herein (*e.g.*, SH2 domain-containing protein-mediated signal transduction-mediated kinase activity, phosphatase activity, or change in phosphotyrosine proteins). It will be appreciated that the degree of modulation provided by a SAP polypeptide or a modulating compound in a given assay will vary, but that one skilled in the art can

determine the statistically significant change or a therapeutically effective change in the level of SH2 domain-containing protein-mediated signal transduction which identifies a SAP family member polypeptide, or identifies a compound which modulates SAP family member polypeptide or is a SAP family member therapeutic.

By "modulating T cell activation" or "altering T cell activation" is meant increasing (*i.e.*, enhancing) or decreasing (*i.e.*, inhibiting) the number of T cells that become stimulated by antigen via their antigen-specific receptors in a given cell population relative to a control cell population not exposed to a test compound. Preferably, the increase or decrease in the given cell population exposed to a test compound is a change of at least 25%, more preferably the change is at least 50%, and most preferably the change is at least one-fold, as compared to a control cell population. T cell activation may be measured by a variety of assays known in the art, including the assays described herein (*e.g.*, T cell activation-mediated upregulation of T cell surface molecules CD69, CD25, and Fas Ligand). Preferably, the cell population is selected from a group including TH1 $CD4^+$ T cells, TH2 $CD4^+$ T cells, and/or $CD8^+$ T cells. It will be appreciated that the degree of modulation provided by a SAP polypeptide or a modulating compound in a given assay will vary, but that one skilled in the art can determine the statistically significant change or a therapeutically effective change in the level of T cell activation which identifies a SAP polypeptide, or identifies a compound which modulates SAP or is a SAP therapeutic.

By "T cell activation" or "antigen-specific T cell activation" is meant a T cell that exhibits an activated phenotype (*e.g.*, increased expression of activation-dependent genes such as interleukin-2, CD69, CD25, γ-interferon, Fas Ligand) in response to stimulation through the antigen-specific T cell receptor/CD3 complex. Antigen-specific T cell activation may be by stimulation of the T cell with a syngeneic antigen-presenting cell presenting the antigen in context with MHC class I or class I. For example, an I-A^b restricted ovalbumin peptide-specific T cell may achieve antigen-specific T cell activation when stimulated with an H-2^b antigen-

presenting cell expressing on its cell surface the ovalbumin peptide in context with class II MHC. Antigen-specific T cell activation may also be achieved by incubating the T cell with an antibody toward CD3 plus an antibody specific toward a costimulatory molecule (*e.g.*, CD28). Antigen-specific T cell activation may be mediated by helper T cells of the Th1, Th2, or Th0 phenotype.

By "antigen" is meant a protein or polypeptide capable of eliciting an immune response. The antigen may be derived from any source.

By "antigen-presenting cell" is meant a cell that expresses on its cell surface MHC proteins. A preferable antigen presenting cell expresses MHC class II proteins on its cell surface, and a most preferable antigen presenting cell expresses both MHC class I and MHC class II proteins on its cell surface.

By "naturally-occurring," as used herein in reference to nucleic acids or polypeptides, is meant sequences found to occur in nature. Included in the definition are naturally-occurring mutations, homologues, isoforms, truncations, splice variants, and other naturally-occurring variants of the nucleic acid or polypeptide.

By "high stringency conditions" is meant conditions that are commonly understood in the art as highly stringent. Exemplary high stringency conditions include hybridizing conditions that employ low ionic strength and high temperature for washing. One, high stringency condition may include hybridization at about 40°C in about 2XSSC and 1%SDS, followed by a first wash at about 65°C in about 2XSSC and 1%SDS, and a second wash at about 65°C in about 1XSSC. Another preferred high stringency condition includes hybridizing at 2X SSC at 40°C with a probe length of at least 40 nucleotides. For other definitions of high stringency conditions, see Ausubel, F. *et al.*, Chapter 6, Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1994 hereby incorporated by reference.

By "protein" or "polypeptide" is meant any chain of more than two amino acids, regardless of post-translational modification such as glycosylation or phosphorylation.

By "pharmaceutically acceptable carrier" means a carrier which is

physiologically acceptable to the treated mammal while retaining the therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable carrier is physiological saline. Other physiologically acceptable carriers and their formulations are known to one skilled in the art and described, for example, in Remington's Pharmaceutical Sciences, (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA.

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%, preferably at least 70%, more preferably at least 85%, still more preferably at least 90%, and most preferably at least 95% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 contiguous amino acids, preferably at least 20 contiguous amino acids, more preferably at least 25 contiguous amino acids, and most preferably at least 35 contiguous amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 contiguous nucleotides, preferably at least 60 contiguous nucleotides, more preferably at least 75 contiguous nucleotides, and most preferably at least 110 contiguous nucleotides.

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Preferably, a polypeptide substantially identical to a reference polypeptide differs only by conservative amino acid substitutions. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "substantially pure polypeptide" is meant a polypeptide that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the

proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is a SAP polypeptide that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure SAP polypeptide may be obtained, for example, by extraction from a natural source (*e.g.*, T lymphocytes); by expression of a recombinant nucleic acid encoding a SAP polypeptide in a cellular system different from the cell from which it naturally originates (*e.g.*, SAP polypeptide expressed in bacteria); or by chemically synthesizing the protein. Purity can be measured by any appropriate method, *e.g.*, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

By "substantially pure nucleic acid" is meant nucleic acid that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant nucleic acid which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic nucleic acid of a prokaryote or eukaryote; or which exists as a separate molecule (*e.g.*, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. The term also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "transgene" is meant any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely exogenous (*i.e.*, foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell which includes a DNA sequence which is inserted by artifice into that cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic mammals (*e.g.*, rodents such as rats or mice) and the DNA (transgene) is inserted by artifice into the nuclear genome.

By "knockout mutation" is meant an alteration in the nucleic acid sequence

that reduces the biological activity of the polypeptide normally encoded therefrom by at least 80% relative to the unmutated gene. The mutation may, without limitation, be an insertion, deletion, frameshift mutation, or a missense mutation. Preferably, the mutation is an insertion or deletion, or is a frameshift mutation that creates a stop codon.

By "transformation" or "transfection" is meant any method for introducing foreign molecules into a cell. Methods for transformation and transfection include, without limitation, lipofection, calcium phosphate precipitation, retroviral delivery, electroporation, and biolistic transformation.

By "positioned for expression" is meant that the desired sequence (*e.g.*, a cDNA) is operably linked to one or more regulatory sequences (*e.g.*, a promoter) which directs transcription and translation of the desired sequence (*i.e.*, facilitates the production of, *e.g.*, a SAP polypeptide, a recombinant protein, or a sense or antisense RNA molecule).

By "operably linked" is meant that a two nucleic acids (*e.g.*, a coding sequence and a gene promoter) are connected such that the sequences have an effect upon each other, where the two sequences are not directly adjacent to each other in the naturally-occurring genome of an organism. For example, a gene promoter from a viral gene operably linked to a coding sequence from a mammalian gene will permit expression of the mammalian protein product encoded by the coding sequence when the appropriate molecules (*e.g.*, transcriptional activator proteins) are bound to the gene promoter sequence. Likewise, a fusion protein can be created by operably linking two coding sequences such that a polypeptide encoded by the first coding sequence is covalently bonded (via a peptide bond) to a polypeptide encoded by the second coding sequence.

By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are cell type-specific, tissue-specific, or inducible by external signals or agents; such elements may naturally occur in the 5' or 3' or intron sequence regions of the native gene, or may

naturally occur in the long terminal repeat (LTR) of a viral genome.

By "reporter gene" is meant any gene which encodes a product whose expression is detectable. A reporter gene product may have one of the following attributes, without restriction: fluorescence (*e.g.*, green fluorescent protein), enzymatic activity (*e.g.*, luciferase or chloramphenicol acetyl transferase), toxicity (*e.g.*, ricin), or an ability to be specifically bound by a second molecule (*e.g.*, biotin or a detectably-labeled antibody).

By "detectably-labeled" is meant any means for marking and identifying a molecule (*e.g.*, an oligonucleotide probe, a cDNA molecule, or an antibody), such that another molecule to which the detectably labeled molecule associates can be detected. Methods for detectably-labeling molecules are well known in the art and include, without limitation, radioactive labeling (*e.g.*, with ^{32}P or ^{35}S) and nonradioactive labeling (*e.g.*, labeling by biotinylation or chemiluminescence with, for example, fluorescein).

By "antisense," as used herein in reference to nucleic acids, is meant a nucleic acid sequence that is complementary to the coding strand of a gene (*e.g.*, a murine or human SAP gene).

By "Th1 helper T cell" is meant a helper T cell (*i.e.*, a $CD4^+$ T cell) which, when stimulated, for example, during an immune response, serves to activate cytotoxic T cells. Th1 cells produce a number of characteristic cytokines including interleukin-2, interleukin-12, and interferon-γ.

By "Th2 helper T cell" is meant a helper T cell (*i.e.*, a $CD4^+$ T cell) which, when stimulated, for example, during an immune response, serves to activate antibody-producing B cells. Th2 cells produce a number of characteristic cytokines including interleukin-4.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 shows the nucleic acid sequence (SEQ ID NO: 1; above) and amino acid sequence (SEQ ID NO: 2; below) of the cytoplasmic domain of SLAM.

Figs. 2A-2D shows the DNA and amino acid sequences of human and murine SAP. Fig. 2A shows the DNA sequence of human SAP (SEQ ID NO: 3). Fig. 2B shows the amino acid sequence of human SAP (SEQ ID NO: 4). Fig. 2C shows the cDNA sequence of murine SAP (SEQ ID NO: 5). Fig. 2D shows the amino acid sequence of murine SAP (SEQ ID NO: 6).

Fig. 3 shows the DNA and protein sequences of the various SAP mutants described herein (SEQ ID Nos: 7-13). Note that in the exon 2 genomic mutant, G is a point mutation identified in A1 patient. In normal individuals, it is a C. Upper case letters in the exon 2 genomic mutant represent exon2 sequence, while lower case letters represent intronic sequence.

Figs. 4A and 4B demonstrate that human SAP contains an SH2 domain. Fig. 4A shows the blocks (in bold-face type) identifying the SH2 domain in the amino acid sequence of human SAP. Fig. 4B shows the five individual SH2 domain-identifying blocks in human SAP.

Fig. 5 shows a comparison of the deduced amino-acid sequences of human SAP and murine SAP with other SH2 domain-containing proteins. Consensus α-helices and β-sheets are indicated in brackets. Exon/intron boundaries, as derived from the murine SAP gene, are demarcated by ↓ arrows. Four truncation mutants were generated in the tail of human SAP (del 1 to 4), and their locations in the amino acid sequence are shown with ⇓ arrows.

Figs. 6A-6D are a series of Western blotting (WB) analyses showing the

characterization of the human and murine SAP proteins and their interactions with SLAM. In Fig. 6A, human T cell tumor cells (Jurkat) and human peripheral blood lymphocytes were lysed and postnuclear lysates (1mg/ml) were immunoprecipitated with a rabbit antiserum directed at human SAP or with a control serum (3 μl). Samples were resolved on SDS-PAGE and immunoblotted with anti-human-SAP (1/1000 dilution). In Fig. 6B, postnuclear lysates (1mg/ml) from C57Bl/6 thymocytes were immunoprecipitated with an anti-murine SAP antibody (3 μl), resolved on SDS-PAGE and immunoblotted with anti-mouse-SAP (1/1000 dilution). In Fig. 6C, SLAM or vector transfected EL-4 cells were cell surface biotinylated and lysed in detergent. Postnuclear lysates (1 mg/ml) were incubated with 5 μg of GST or GST-SAP for 1 hour in the presence of glutathione beads or 1 μg of anti-SLAM antibodies (2E7) for 3 hours in the presence of protein G beads. Bead associated proteins were extracted, SDS-PAGE resolved, and immunoblotted with streptavidin-HRP. In Fig. 6D, human peripheral blood lymphocytes (PBL) were activated with PHA for 5 days, and then lysed ($1x10^8$/ml) and immunoprecipitated with an anti-SLAM monoclonal antibody (2E7) or with a control antibody. Immunoprecipitates were resolved on SDS-PAGE and immunoblotted with a anti-SLAM serum (upper panel) or with a rabbit anti-SAP antibody (lower panel).

Figs. 7A-7C are Northern blotting analyses showing SAP expression using a ^{32}P radiolabelled human SAP probe. Fig. 7A shows a Northern blotting analysis of poly A+ mRNA isolated from various human tissues (Clontech). A human β-actin probe was used as loading control. Fig. 7B shows a Northern blotting analysis of total RNA from different human and mouse cell lines (20 μg/lane). A human 18S rRNA probe was used as a loading control. Fig. 7C shows a Northern blotting analysis of human T cell subsets and two EBV+ human B cell lines. A human β-actin probe was used as loading control.

Figs. 8A and 8B show SAP mRNA analyses of XLP patients. Fig. 8A is a

photograph of a 10% polyacrylamide gel in which are resolved cDNAs isolated using RT PCR (reverse transcriptase-PCR) from PBMC (peripheral blood mononuclear cells) from XLP patients and healthy donors. Samples A1, B1 and B2 are from XLP patients; sample B3 is from a healthy brother of B1 and B2; and samples CT-1 and CT-2 are from healthy controls. Fig. 8B is a Northern blotting analysis of total RNA from human T cell tumor cells (Jurkat), from a subset of $CD45RA^{High}$ cells from a healthy donor, and from PBMC from patient B2 (20 μg/lane). Specific RNA were detected using a ^{32}P radiolabelled human SAP probe; β-actin probe was used as a loading control.

Figs. 9A and 9B show the nucleic acid and amino acid sequences of SAP isolated from a patient with X-linked proliferative disease (XLP). DNA products were cloned in the pCR2.1 vector, and the nucleotide sequence determined on two cDNA clones in both directions using an ABI prism 377 DNA sequencer. Fig. 9A shows a comparison of the nucleic acid sequence of two cDNA clones isolated by RT-PCR from an XLP patient (A1-1 and A1-2) with those of human SAP (hSAP and hSAPΔ55). Fig. 9B shows a comparison of the predicted amino acid sequences of SAP protein isolated from an XLP patient and of SAP protein isolated from a normal human. SAP.

Figs. 10A-10C show genomic analysis of patients with X-linked proliferative disease. Fig. 10A depicts the location of the point mutation near exon 2 of XLP patient A1, as well as a mechanism to explain the generation of the variant form of SAP, hSAPΔ55, found in all healthy individuals. Fig. 10B is a photograph of a 10% polyacrylamide gel in which are resolved Mnl I-digested PCR amplified products of Exon 2 sequence from genomic DNA from patient A1, three healthy individuals (CT-1, CT-2, and B3), and two human cell lines (Raji and Jurkat). In addition, PCR products from 78 healthy women and 30 healthy men were analyzed. Fig. 10C is a photograph of a 2% agarose gel in which are resolved PCR amplified products of

hSAP Exon 1, Exon 2, Exon 3, Exon 4, and BRCA1 Exon 2 from genomic DNA from patient A1, B1, B2, B3, and from the cell line Raji.

Figs. 11A and 11B show the CD8SLAM fusion proteins. Fig. 11A shows schematic diagrams of SLAM3, SLAM4, CD8-SLAM3 fusion protein, CD8-SLAM4 del 1 fusion protein, and CD8-SLAM4 fusion protein. Tyrosine residues in the cytoplasmic domains of these proteins are as indicated. Fig. 11B shows the amino acid sequences of the cytoplasmic domains of SLAM3 and SLAM4, as well as the amino acid sequence of a truncated cytoplasmic domain of the SLAM4 del 1 protein.

Figs. 12A-12D are a series of Western blotting (WB) analyses showing that the SAP SH2-domain binds to a specific sequence in the cytoplasmic domain of SLAM. In Fig. 12A, COS-7 cells were transfected with the indicated constructs, biotinylated, and lysed. Postnuclear lysates were immunoprecipitated with anti-CD8 (OKT8) or anti-SLAM antibodies, and immunoprecipitates were immunoblotted with anti-human-SAP rabbit sera (upper panel) or streptavidin (lower panel). In Fig. 12B, postnuclear mouse thymocytes lysates (1mg/ml) were incubated for 1 hour with the indicated peptides coupled to beads (7 μM) or control beads in the absence or presence of free peptides (280 μM). Bead- associated proteins were extracted and immunoblotted with anti-human SAP rabbit serum (1/1000 dilution). In Fig. 12C, postnuclear mouse thymocytes lysates (1 mg/mL) were incubated for 1 hour with SLAM Y1 peptide coupled to beads (7 mM) in the absence or presence of free peptides (70 μM). Bead associated proteins were extracted and immunoblotted with an anti-murine-SAP rabbit serum (1/1000 dilution). In Fig. 12D, COS-7 cells were co-transfected with CD8-SLAM3 construct and one of the following SAP deletion mutants cloned into the pCMV2-FLAG construct: Del 1, Del 2, Del 3 or Del 4 (see Fig. 5). Cell surface proteins were biotinylated, and cells were lysed 48 hours after transfection. Postnuclear lysates were immunoprecipitated with 1 μg of anti-CD8 antibody (OKT8) and immunoprecipitates were immunoblotted with anti-FLAG

antibody (KODAK, 1/1000 dilution) (upper panel) or streptavidin (lower panel).

Figs. 13A-13D are a series of Western blotting (WB) analyses showing that SAP blocks recruitment of the tyrosine phosphatase SHP-2 to the phosphorylated cytoplasmic domain of SLAM. COS-7 cells were transfected as indicated, cell surface biotinylated and lysed. In Fig. 13A, postnuclear lysates were immunoprecipitated with anti-CD8 antibodies to isolate the CD8-SLAM3 chimera, and immunoprecipitates were immunoblotted with either streptavidin (upper panel) or anti-FLAG antibody (lower panel). In Fig. 13B, postnuclear lysates were incubated for 1 hour with the SLAM Y1 peptide coupled to beads (7μM). Bead- associated proteins were extracted and immunoblotted with anti-FLAG antibody (1/1000 dilution). In Figs. 13C and 13D, COS-7 cells were co-transfected with a combination of SLAM, SAP, and *c-fyn* constructs. Cell surface expressed proteins were biotinylated 48 hours after transfection, and cells were lysed. Postnuclear lysates were immunoprecipitated with anti-SLAM antibodies and immunoprecipitates were immunoblotted with anti-Phosphotyrosine-HRP (Zymed Laboratories, San Francisco, CA), streptavidin-HRP (Zymed), rabbit anti-SHP2 (Santa Cruz Biotech., Santa Cruz, CA) or anti-human SAP rabbit sera. Note that in these figures, SLAM is seen as a broad band because of its extensive glycosylation (Cocks *et al.*, *supra*).

Fig. 14 is a bar graph demonstrating that SAP has a positive affect in the SLAM co-stimulatory pathway. Jurkat human T cells were transfected with an IL-2-promoter luciferase reporter construct plus SLAM, SAP, SLAM+SAP constructs, or vector only (pCDNA3). Luciferase activity was measured after stimulation with antibodies as indicated (hatched bar, no antibody; black bar, anti-SLAM, white bar; anti-CD3 plus anti-SLAM, gray bar).

Fig. 15 shows the DNA sequence of the 5' region/exon1/intron1 of the murine SAP gene (SEQ ID NO: 14).

Fig. 16 shows the DNA sequence of exon2/intron2 of the murine SAP gene (SEQ ID NO: 15).

Fig. 17 shows the DNA sequence of intron2/exon3/intron3/exon4/intron4 of the murine SAP gene (SEQ ID NO: 16).

Fig. 18 is a schematic diagram showing the map locations of the four murine SAP exons on the murine X chromosome.

Fig. 19 is a schematic diagram showing putative transcription factor binding sites on the human and murine SAP gene sequences.

Fig. 20 is a Northern blotting analysis using as a probe radiolabelled murine SAP cDNA showing the level of murine SAP mRNA species (the 0.9 kB band) in murine Th1 helper T cells (as identified by the presence of IFN-γ mRNA) or Th2 helper T cells (as identified by the presence of IL-4 mRNA) following 6 hours of stimulation with anti-CD3 antibody plus human IL-2. Equivalent loading of the lanes is shown in the equivalent amounts of the 2.2 kB β-actin mRNA per lane.

Fig. 21 is a Western blotting analysis of anti-CD8 immunoprecipitations from externally biotinylated COS cells that had been transiently transfected with CD8SLAM3 + empty vector; CD8SLAM3 + Flag-tagged EAT-2, and CD8SLAM3 + Flag-tagged SAP. The upper panel shows the results obtained by Western blotting with anti-FLAG antibody; the middle panel shows the results obtained by blotting with anti-SAP antibody; and the lower panel shows blotting with streptavidin.

Detailed Description

We have identified the T cell protein, SAP, as the gene that is altered in patients with X-linked proliferative disease (XLP). Not only has SLAM been

discovered to be the cell surface protein to which the XLP gene product (*i.e.*, SAP) binds, but a novel T cell signal transduction pathway initiated by the co-receptor molecule SLAM has also been revealed. Furthermore, we have identified the SAP family of proteins involved in signal transduction regulation, which includes not only the novel SAP protein described herein, but also the EAT-2 protein (Thompson *et al.*, Oncogene 13: 2649-2658, 1996).

Binding of a SAP family member protein to the cytoplasmic domain of SLAM blocks the recruitment of SHP-2. Therefore two modes of SLAM signaling are likely to exist: one in which the inhibitor SAP family member acts as a negative regulator and another in which the SHP-2 dependent signal transduction pathway becomes operational. A switch between these two signalling scenarios could occur upon T cell activation, because the level of expression of the SAP protein-encoding gene was observed to diminish rapidly after triggering of the T cell receptor. It is also possible that upon activation of T cells, SAP is released from SLAM and subsequently binds to other protein(s). The concept that SAP family members act as an inhibitory molecules of SH2-domain interactions can, in general, be extended to molecules other than SLAM. The studies described herein support the model that SAP family members control signal transduction pathways that are initiated by interactions between SLAM molecules on the interface between T and B cells.

The failure of the immune system to eliminate EBV-infected B cells in XLP appears not to be caused by a B-cell-specific defect, but rather by defective EBV specific helper T cell and cytotoxic T cell responses (Lanyi *et al.*, Genomics 39: 55-56, 1997, Article Number GE964466; Purtilo, D.T., Immunology Today 4: 291-295, 1983; Seemayer *et al.*, Pediatr. Res. 38: 471-478, 1995; Sutkowski *et al.*, J. Exp. Med. 184: 971-800, 1996). As the SAP protein (*i.e.*, one of the two members of the SAP family) is found predominantly in thymus derived lymphocytes, mutations in SAP protein will most likely affect SLAM induced signal transduction events in T lymphocytes. Many XLP patients display impaired interferon-γ production by helper T cells, suggesting a Th2 like phenotype.

Because engagement of SLAM during antigen-specific T-cell stimulation has been shown to induce IFN-γ production and to redirect the Th2 phenotype to a Th1/Th0 phenotype (Carballido *et al.*, J. Immunol. 159: 4316-4321, 1997), insufficient responses of the T helper subset in XLP patients, could result from an impaired function of the SLAM / SAP pathway. Alternatively, since reduced EBV specific cytotoxic T cell responses have been reported in XLP patients, it is possible that EBV infected B cells cannot be "licensed" to activate T-killer cells by helper T cells that lack SAP (Ridge *et al.*, Nature 393: 474-478, 1998; Schoenberger *et al.*, Nature 393: 480-483, 1998; Bennett *et al.*, Nature 393: 478-480, 1998). In the latter model, the SLAM/SLAM binding in T/B lymphocyte interactions might serve the same purpose as CD40/CD40L interactions in activation of dendritic cells by helper T cells. Because of the results presented herein, and, in particular, because of the correlation between the XLP phenotype and the lack of binding of the respective SAP family member protein mutants to its docking site on SLAM, we conclude that a dysfunction in SLAM/SAP family member induced signal transduction pathways is likely to be the underlying cause for the inability to generate an effective T cell response capable of sustained elimination of EBV infected B cells in XLP patients.

Uncontrolled B cell proliferation in XLP patients frequently leads to non-Hodgkin's lymphomas induced by EBV. This type of non-Hodgkin lymphoma has been detected with increasing frequencies in AIDS patients, bone marrow transplant patients, and individuals who undergo T cell suppressive treatment for other reasons. Some of these disease states might be caused by abnormal regulation of a SAP family member protein-encoding gene and/or the T cell subsets that express SAP protein.

I. SAP Family Member Proteins' Biological Activity

SAP protein was identified by virtue of its ability to bind to the cytoplasmic tail of SLAM4 in a yeast two-hybrid screen. Interestingly, although tyrosine residues which are normally phosphorylated in mammalian cells are not phosphorylated in yeast cells, an SH2 domain is present in SAP protein. However, as the SH2 domain

in SAP protein was also found to be able to bind phosphorylated tyrosine residues, SAP binds to both non-phosphorylated and phosphorylated tyrosine residues.

Tyrosine phosphorylation of SLAM gives rise to its being bound by the protein tyrosine phosphatase, SHP2 (also known as SHPTP-2 or syp-2). The SLAM-SHP2 complex can act as a negative regulator of signal transduction cascades (Marengere *et al.*, Science 272: 1170-1173, 1996). Binding of non-tyrosine phosphorylated SLAM by SAP protein prevents SHP2 from binding and, hence, enhances SLAM's contribution to antigen-specific T cell activation.

Using SAP protein as the prototype, we also identified another member of the SAP family, namely EAT-2 (Thompson *et al.*, Oncogene 13: 2649-2658, 1996) which also binds the cytoplasmic tail of SLAM and, thus, modulated SH2 domain-containing protein-mediated signal transduction.

While antigen-specific T cell activation is enhanced by the upregulation of the expression of a SAP family member protein, given the unique ability of a SAP family member protein to bind both phosphorylated tyrosine residues and non-phosphorylated tyrosine residues, expression of SAP family member protein in a non-T cell may either enhance or inhibit SH2 domain-containing protein-mediated signal transduction in that cell. Whether expression of a SAP family member enhances or inhibits SH2 domain-containing protein-mediated signal transduction in any particular cell depends upon which SH2 domain-containing proteins are involved in any particular signalling pathway.

Identification of the SAP family allows the characterization and exploitation of SAP family members' biological activity which is useful for modulating SH2 domain-containing protein-mediated signal transduction cellular events. For example, administration of a SAP family member protein, or polypeptide fragment thereof, may enhance antigen-specific T cell activation, as measured by antigen-specific T cell activation assays known in the art and described herein. An antigen-specific T cell activation-inhibiting amount of a SAP reagent (*e.g.*, a compound that reduces the biological function of a SAP family member, such as an anti-SAP protein neutralizing

antibody or SAP protein antisense nucleic acid) may be similarly assessed. Such assays may be carried out in a cell which either expresses endogenous SAP family member proteins, or a cell into which is introduced an ectopic SAP family member polypeptide. Preferably, the cell is a T cell that is capable of undergoing antigen-specific T cell activation.

Another approach to characterize and exploit the activity of a SAP family member (*e.g.*, SAP protein) in antigen-specific T cell activation utilizes the activation of the nuclear transcription factor NFAT in TCR-mediated signal transduction. In this system the role of a SAP family member protein in NFAT activation may be readily elucidated in various assays known to the skilled artisan. For example, one method of rapidly measuring NFAT activity is through the use of a reporter gene whose expression is directed by a NFAT binding site containing promoter (Rooney *et al.*, Mol. Cell. Biol. 15: 6299-6310 1995; Luo *et al.*, J. Exp. Med. 184: 141-147, 1996). The expression vector is preferably inserted by artifice into a cell capable of undergoing antigen-specific T cell activation or is responsive to TCR-mediated signal transduction. By detecting a change in the level of expression of the reporter gene, an NFAT-inducing or inhibiting ability of a SAP family member polypeptide or reagent may be readily assessed.

These analyses may be undertaken with human or mouse SAP or EAT-2, or other SAP family member protein (*e.g.*, an isolated SH2 domain). Likewise, SAP family members' biological activity may be assayed in any cell type that has an SH2 domain-containing protein-mediated signal transduction pathway. For example, ligation of platelet derived growth factor (PDGF) with the PDGF receptor present on a fibroblast results in activation of the protein tyrosine kinase activity of the PDGF receptor. An effect of an introduced SAP family member polypeptide on PDGF receptor-mediated signaling can be assessed by a number of different ways, including changes in fibroblast cell cycle, and changes in the association of a number of SH2 domain-containing proteins with the phosphorylated PDGF receptor, such as phosphatidylinositol 3-kinase (PI-3 kinase) and phospholipase C-γ (PLC-γ).

The ability of SAP family member proteins to modulate SH2 domain-containing protein-mediated signal transduction can be defined in *in vitro* systems in which alterations SH2 domain-containing protein-mediated signal transduction can be detected. For example, once a SAP family member polypeptide is ectopically expressed in a cell, SH2 domain-containing protein-mediated signal transduction can be induced in that cell by standard methods, for example, by addition of ligand to a cell whose SH2 domain-containing protein-mediated signal transduction pathway is initiated by that the ligand receptor on the cell surface (*e.g.*, addition of PDGF to a PDGF receptor-expressing fibroblast). As a control, cells are cultured under the same conditions as those induced to undergo SH2 domain-containing protein-mediated signal transduction, but either not transfected, or transfected with a vector that lacks a SAP family member-encoding insert. The ability of each SAP family member encoding construct to enhance or inhibit SH2 domain-containing protein-mediated signal transduction upon expression can be quantified by calculating the activation profiles of the cells, *i.e.*, the ratio of the increase in the number of transfected cells to the increase in the number of control cells. These experiments can confirm the presence of SH2 domain-containing protein-mediated signal transduction modulating activity of a full length SAP family member protein protein. These assays may also be performed in combination with the application of additional compounds in order to identify compounds that modulate SH2 domain-containing protein-mediated signal transduction via expression of the SAP family member protein.

II. Characterization, Identification, and Synthesis of SAP Family Member Proteins and Polypeptides

SAP family member polypeptides may be synthesized by introducing the polypeptide-encoding nucleic acid sequences, or fragments thereof, into various cell types or using *in vitro* extracellular systems. The function of SAP family member proteins may then be examined under different physiological conditions. For example, a SAP family member polypeptide-encoding cDNA sequence may be

manipulated to characterize the expression of a given SAP family member in a particular cellular compartment. Alternatively, cell lines may be produced which over-express the SAP family member gene product allowing purification of SAP family member polypeptides for biochemical characterization, large-scale production, antibody production, and patient therapy (*e.g.*, therapy of XLP patients).

For protein expression, eukaryotic and prokaryotic expression systems may be generated in which SAP family member encoding nucleic acid sequences are introduced into a plasmid or other vector which is then used to transform living cells. Constructs in which SAP family member encoding nucleic acids containing the entire open reading frames are inserted in the correct orientation into an expression plasmid may be used for protein expression. Alternatively, portions of the SAP family member gene sequences, including wild-type or mutant SAP family member sequences, may be inserted. Prokaryotic and eukaryotic expression systems allow various important domains of the SAP family member proteins to be recovered as fusion proteins and then used for binding, structural and functional studies, and also for the generation of appropriate antibodies. Since expression of a SAP family member protein modulates signal transduction involving SH2 domain-containing proteins, it may be desirable to put the expression of a SAP family member protein under the control of an inducible promoter.

Typical expression vectors contain promoters that direct the synthesis of large amounts of mRNA corresponding to the inserted SAP family member-encoding nucleic acid in the plasmid bearing cells. They may also include eukaryotic or prokaryotic origin of replication sequences allowing for their autonomous replication within the host organism, sequences that encode genetic traits that allow vector-containing cells to be selected for in the presence of otherwise toxic drugs, and sequences that increase the efficiency with which the synthesized mRNA is translated. Stable long-term vectors may be maintained as freely replicating entities by using regulatory elements of, for example, viruses (*e.g.*, the OriP sequences from the Epstein Barr Virus genome). Cell lines may also be produced which have integrated

the vector into the genomic DNA, and in this manner the nucleic acid product is produced on a continuous basis.

Expression of foreign sequences in bacteria such as *Escherichia coli* requires the insertion of the SAP family member encoding nucleic acid sequence into a bacterial expression vector. This plasmid vector contains several elements required for the propagation of the plasmid in bacteria, and expression of inserted DNA of the plasmid by the plasmid-carrying bacteria. Propagation of only plasmid-bearing bacteria is achieved by introducing in the plasmid selectable marker-encoding sequences that allow plasmid-bearing bacteria to grow in the presence of otherwise toxic drugs. The plasmid also bears a transcriptional promoter capable of producing large amounts of mRNA from the cloned DNA. Such promoters may or may not be inducible promoters which initiate transcription upon induction. The plasmid also preferably contains a polylinker to simplify insertion of the DNA in the correct orientation within the vector.

In vitro expression of SAP family member proteins, fusions, polypeptide fragments, or mutants encoded by cloned DNA is also possible using the T7 late-promoter expression system in *E. coli* (see standard protocol in, *e.g.*, Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1994). Using this system, large amounts of mRNA corresponding to the cloned SAP family member encoding DNA can be produced, and the resulting mRNA or protein can be radioactively labeled according to standard techniques.

Once the appropriate expression vectors containing a SAP family member encoding nucleic acid, or fragment, fusion, or mutant thereof, are constructed, they may be introduced into an appropriate host cell. The precise host cell used is not critical to the invention. A wide variety of expression systems may be used to produce the recombinant SAP family member proteins. Thus, sequences encoding SAP family member proteins may be introduced into a prokaryotic host (*e.g.*, *E. coli*) or in a eukaryotic host (*e.g.*, *S. cerevisiae*, insect cells such as Sf9 cells, or mammalian cells such as COS, NIH 3T3, Daudi, Jurkat, or HeLa cells). These cells

are commercially available from, for example, the American Type Culture Collection (ATCC), Manassas, VA. The method of transformation and the choice of expression vehicle (*e.g.*, expression vector) will depend on the host system selected. For example, for expression in insect cells, the baculovirus system may be used (as commercially available from, *e.g.*, Clontech, Palo Alto, CA); for expression in mammalian cells, the vaccinia virus system may be used (as described, *e.g.*, in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1994). If desired, the expression system may be used in conjunction with other protein expression techniques, for example, the myc tag approach described by Evan *et al.* (Mol. Cell Biol. 5:3610-3616, 1985).

Eukaryotic expression systems permit appropriate post-translational modifications to expressed SAP family member proteins, or mutants or fragments thereof. Thus, expression in eukaryotic cells enables the study of the function of the normal complete protein, specific portions of the protein, or of naturally-occurring polymorphisms and artificially produced mutated proteins. Eukaryotic cell expression also allows the identification of regulatory elements located in the 5', 3', or intronic regions of SAP family member genes, and their roles in tissue regulation of protein expression. For example, SAP family member proteins may be produced by a transiently-transfected or stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public (*e.g.*, see Pouwels *et al.*, Cloning Vectors: A Laboratory Manual, 1985, Supp. 1987), as are methods for constructing such cell lines (see *e.g.*, Ausubel *et al.*, *supra*). Eukaryotic cells expressing SAP family member proteins may also be used to test the effectiveness of pharmacological agents on, for example, SAP family member-associated antigen-specific T cell activation, or as means by which to study SAP family member proteins as components of other SH2 domain-containing protein-mediated signal transduction systems.

Once the recombinant protein is expressed, it can be isolated from the expressing cells by cell lysis followed by protein purification techniques, such as

affinity chromatography. In this example, an anti-SAP family member antibody, which may be produced by the methods described herein, can be attached to a column and used to isolate the recombinant SAP family member proteins. Lysis and fractionation of SAP protein-harboring cells prior to affinity chromatography may be performed by standard methods (see *e.g.*, Ausubel *et al.*, *supra*). Once isolated, the recombinant protein can, if desired, be purified further by *e.g.*, by high performance liquid chromatography (HPLC; *e.g.*, see Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, Work and Burdon, Eds., Elsevier, 1980).

The SAP family member-encoding nucleic acid sequences can be altered using procedures known in the art, such as restriction endonuclease digestion, DNA polymerase fill-in, exonuclease deletion, terminal deoxynucleotide transferase extension, ligation of synthetic or cloned DNA sequences, and site-directed sequence alteration using specific oligonucleotides together with PCR. Polypeptides of the invention, particularly short SAP family member polypeptide fragments, such as the fragment corresponding to the SAP protein SH2 domain of approximately 100 amino acids in length, can also be produced by chemical synthesis (*e.g.*, by the methods described in Solid Phase Peptide Synthesis, 2nd ed., 1984, The Pierce Chemical Co., Rockford, IL). These general techniques of polypeptide expression and purification can also be used to produce and isolate useful SAP family member polypeptide fragments or analogs, as described herein.

III. SAP Family Member Polypeptide Fragments

Polypeptide fragments including various portions of SAP family member proteins are useful in identifying the domains important for the biological activities of SAP family member proteins. Such fragments may be generated by expression of SAP family member polypeptide fragment encoding nucleic acid fragments, generated using the nucleotide sequences provided herein, or by chemical synthesis.

SAP family member polypeptide fragments are useful, for example, in evaluating the portions of the SAP family member protein (*e.g.*, the SH2 domain)

involved in SH2 domain-containing protein-mediated signal transduction. In one example, polypeptide fragments of SAP family members are used to induce or prevent activation-dependent gene expression with or without antigen stimulation through the T cell receptor. T cell activation assays are known in the art, and include, without limitation, expression of activation-dependent genes, such as the cell surface receptors CD25, CD69, and FAS ligand, and production of cytokines, such as interleukin-2 (IL-2) and interferon-γ (IFN-γ). Preferably, such SAP family member polypeptide fragments may include the SAP:SLAM or the EAT2:SLAM binding domain, or the SAP protein SH2 domain.

In another example, polypeptide fragments of SAP family members are used to induce or inhibit an SH2 domain-containing protein-mediated signal transduction event in a non-lymphoid cell. SAP family member polypeptide fragment activities are assessed using one or more known assays which depend upon the cell type and the pathway in which the SH2 domain-containing protein-mediated signal transduction event plays a role. For example, a SAP fragment-mediated modulation of cell proliferation induction by an SH2 domain-containing protein-mediated signal transduction event (*e.g.*, ligation of the epidermal growth factor (EGF) receptor by EGF) may be readily assayed in epidermal cell proliferation assays by modifying known cell proliferation techniques (see, *e.g.*, Ausubel *et al.*, *supra*). In T cells, SAP family member polypeptide fragments may also be used to inhibit normal activities of an endogenous full-length SAP family member in a cell exhibiting an inappropriately high level of activity of a SAP family member protein (*e.g.*, inappropriately high level of SAP protein activity in a T cell of a patient with an autoimmune disease).

IV. SAP Family Member Antibodies

In addition to the rabbit polyclonal antibodies that recognize human SAP protein and murine SAP described below, monoclonal antibodies directed toward SAP family member proteins (*e.g.*, human EAT-2) may be produced by using as antigen a SAP family member protein isolated from SAP family member-expressing

cultured prokaryotic or eukaryotic cells or a SAP family member isolated from expresing cells (*e.g.*, EAT-2 from Ewing's sarcoma cells). Methods for generating monoclonal antibodies are well known in the art and are described, for example, in Kohler *et al.*, Nature 256: 495, 1975; Kohler *et al.*, Eur. J. Immunol. 6:511, 1976; Kohler *et al.*, Eur. J. Immunol. 6: 292, 1976; Hammerling et al., Monoclonal Antibodies and T Cell Hybridomas, Elsevier, New York, NY, 1981; and Coligan *et al.*, Current Protocols in Immunology John Wiley & Sons, New York, NY, 1994. Once produced, monoclonal antibodies may be tested for specific SAP family member protein recognition by standard Western blotting or immunoprecipitation analyses.

Antibodies that specifically recognize SAP family member proteins may be produced using amino acid sequences that do not reside within highly conserved regions, and that appear likely to be antigenic, as analyzed by criteria such as those provided by the PeptideStructure Program (Genetics Computer Group Sequence Analysis Package, Program Manual for the GCG Package, Version 7, 1991) using secondary structure according to either the Chou-Fasman method (Adv. in Enzymol. 47: 45-148, 1978) or the Garnier-Osguthorpe-Robson method (Garnier *et al.*, J. Mol. Biol. 120: 97, 1978); hydrophilicity according to either the Kyte-Doolittle method or Hopp-Woods method (Proc. Natl. Acad. Sci. USA 78: 3824-3828, 1981); surface probability according to the Emini method (Emini *et al.*, J. Virol., 55: 836-839, 1985); flexibility according to the Karplus-Schulz method; and the antigenic index according to the Jameson-Wolf method (CABIOS 4(1): 181-186, 1988). Such SAP sequences preferably reside in the short cytoplasmic tail of SAP (*i.e.*, the final 26 amino acid residues of human SAP, the final 24 amino acid residues of murine SAP, or the final 32 amino acids of murine EAT-2). Conversely, antibodies may be produced using SAP amino acid sequences that reside within highly conserved regions. For example, amino acid sequences from the SH2 domain of SAP may be used as antigen to generate antibodies specific toward human or murine SAP.

In addition to intact monoclonal and polyclonal anti-SAP family member

antibodies, various genetically engineered antibodies, humanized antibodies, and antibody fragments, including F(ab')2, Fab', Fab, Fv, and sFv fragments, may generated that specifically recognize human or murine SAP protein, human or murine EAT-2 protein, or fragments thereof. Antibodies can be humanized by methods known in the art (*e.g.*, by expression in transgenic animals, as described in Green *et al.*, Nature Genetics 7: 13-21, 1994). In addition, monoclonal antibodies with a desired binding specificity can be commercially humanized (Scotgene, Scotland; Oxford Molecular, Palo Alto, CA).

In addition, Ladner (U.S. Patent Nos. 4,946,778 and 4,704,692) describes methods for preparing single polypeptide chain antibodies. Ward *et al.* (Nature 341: 544-546, 1989) describe the preparation of heavy chain variable domains, which they term "single domain antibodies," which have high antigen-binding affinities. McCafferty *et al.* (Nature 348: 552-554, 1990) show that complete antibody V domains can be displayed on the surface of fd bacteriophage, that the phage bind specifically to antigen, and that rare phage (one in a million) can be isolated after affinity chromatography. Boss *et al.* (U.S. Patent 4,816,397) describe various methods for producing immunoglobulins, and immunologically functional fragments thereof, which include at least the variable domains of the heavy and light chain in a single host cell. Cabilly *et al.* (U.S. Patent 4,816,567) describe methods for preparing chimeric antibodies.

V. Identification of Compounds that Modulate Expression of SAP Family Member Proteins

Based on our experimental results, we have developed a number of screening procedures for identifying therapeutic compounds (*e.g.*, that modulate SH2 domain-containing protein-mediated signal transduction) which can be used in human patients. In one particular example, compounds that upregulate expression of SAP family member proteins are considered useful for the treatment of patients with XLP. Likewise, compounds that downregulate SAP family member protein expression are

useful in treating patients with diseases hallmarked by an excessive amount of antigen-specific T cell activation (*e.g.*, autoimmune diseases). In general, the screening methods of the invention involve screening any number of compounds for therapeutically active agents by employing any number of *in vitro* or *in vivo* experimental systems.

The methods of the invention simplify the evaluation, identification, and development of active agents for the treatment and prevention of conditions involving an inappropriate level of SH2 domain-containing protein-mediated signal transduction, which may be excessive or insufficient, depending upon the condition. These screening methods provide a facile means for selecting natural product extracts or compounds of interest in a large population, which are further evaluated and condensed to a few active and selective materials. Constituents of this pool are then purified and evaluated to determine their SH2 domain-containing protein-mediated signal transduction inhibiting or inducing activities.

In general, novel drugs for the treatment of conditions involving an inappropriate level of SH2 domain-containing protein-mediated signal transduction are identified from large libraries of both natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic-, or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (*e.g.*, semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich

Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art. Furthermore, any library or compound may be readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development understand that methods for dereplication (*e.g.*, taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their SH2 domain-containing protein-mediated signal transduction modulating activities should be employed whenever possible.

When a crude extract is found to have SH2 domain-containing protein-mediated signal transduction inhibiting or inducing activities, or both, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having SH2 domain-containing protein-mediated signal transduction modulating activities. The same *in vivo* and *in vitro* assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art. Compounds shown to be useful agents for the treatment of pathogenicity may be chemically modified according to methods known in the art. Compounds identified as being of therapeutic value are subsequently analyzed using any standard animal model of degenerative disease or cancer known in the art.

Below we describe screening methods for identifying and evaluating the

efficacy of a compound as a SH2 domain-containing protein-mediated signal transduction modulating agent, and in particular, an antigen-specific T cell activation inhibiting or inducing agent. These methods are intended to illustrate, not limit, the scope of the claimed invention.

a) Screens for Compounds Affecting SAP Family Member Protein Expression

SAP family member protein encoding DNAs may be used to facilitate the identification of compounds that increase or decrease SAP family member protein expression. In one approach, candidate compounds are added, in varying concentrations, to the culture medium of cells expressing SAP family member mRNA. The SAP mRNA expression is then measured, for example, by Northern blotting analysis (Ausubel *et al.*, *supra*) using a SAP family member protein encoding DNA, cDNA, or RNA fragment as a hybridization probe. The level of SAP family member protein encoding mRNA expression in the presence of the candidate compound is compared to the level of mRNA expression of a SAP family member protein in the absence of the candidate compound, all other factors (*e.g.*, cell type and culture conditions) being equal.

The effect of candidate compounds on SAP family member protein modulation of SH2 domain-containing protein-mediated signal transduction may, instead, be measured at the level of translation by using the general approach described above with standard protein detection techniques, such as Western blotting or immunoprecipitation with an antibody specific toward a SAP family member protein (for example, the SAP protein-specific antibody described herein).

In an alternative approach to detecting compounds which regulate SAP at the level of transcription, candidate compounds may be tested for an ability to regulate a reporter gene whose expression is directed by a SAP family member-encoding gene promoter. For example, a cell which does not express a SAP family member may be transfected with a expression plasmid, such as a luciferase reporter gene operably linked to the SAP family member-encoding gene promoter. Candidate compounds may then be added, in varying concentrations, to the culture medium of the cells.

Luciferase expression levels may then be measured, for example, using the luciferase assay system kit used herein that is commercially available from Promega (Madison, WI), and rapidly assessing the level of luciferase activity on a luminometer. The level of luciferase expression in the presence of the candidate compound is compared to the level of luciferase expression in the absence of the candidate compound, all other factors (*e.g.*, cell type and culture conditions) being equal.

Compounds that modulate the level of expression of a SAP family member protein may be purified, or substantially purified, or may be one component of a mixture of compounds such as an extract or supernatant obtained from cells, from mammalian serum, or from growth medium in which mammalian cells have been cultured (Ausubel *et al.*, *supra*). In an assay of a mixture of compounds, expression of a SAP family member protein is tested against progressively smaller subsets of the compound pool (*e.g.*, produced by standard purification techniques such as HPLC or FPLC) until a single compound or minimal number of effective compounds is demonstrated to modulate expression of a SAP family member protein.

b) Screens for Compounds Affecting SAP Family Member Protein Biological Activity

Compounds may also be screened for their ability to affect the ability of a SAP family member protein to modulate, for example, SH2 domain-containing protein-mediated signal transduction. In this approach, the degree of SH2 domain-containing protein-mediated signal transduction in the presence of a candidate compound is compared to the degree of SH2 domain-containing protein-mediated signal transduction in its absence, under equivalent conditions. Again, the screen may begin with a pool of candidate compounds, from which one or more useful modulator compounds are isolated in a step-wise fashion. The level of an SH2 domain-containing protein mediated signal transduction pathway may be measured by any standard assay. In particular, the SH2 domain-containing protein-mediated antigen-specific T cell activation signaling pathway may be measured using T cell activation assays, many of which are described herein.

Another method for detecting compounds that modulate the SH2 domain-containing protein mediated signal transduction modulating activity of a SAP family member protein is to screen for compounds that interact physically with a given SAP family member polypeptide in a yeast two-hybrid system, as described below.

Another method for detecting protein interactions is an *in vitro* binding assay. In this method, one protein, which may be recombinantly produced and may be a fusion protein, is used like an antibody in an immunoprecipitation reaction to bind to and remove its interacting protein partner from a sample, such as a cell lysate. For example, the GST-SAP fusion protein described below may be recombinantly produced in bacteria and used to isolate a compound capable of binding a SAP family member. Resolution of the bead-bound proteins by SDS-PAGE will detect a SAP family member interacting protein, which may then be sequenced by N-terminal peptide sequencing.

A compound that promotes an increase in the expression or biological activity of the SAP family member protein is considered particularly useful in the invention; such a molecule may be used, for example, as a therapeutic to increase cellular levels of a SAP family member and thereby exploit the ability of SAP family member polypeptides to modulate SH2 domain-containing protein mediated signal transduction. For example, a compound that can increase the level of a SAP family member protein and thereby induce the SAP family member protein-mediated enhancement of antigen-specific T cell activation would be advantageous in the treatment of diseases involving insufficient antigen-specific T cell activation (*e.g.*, the XLP disease, tuberculosis, AIDS, schistosomiasis) or cancer (*e.g.*, breast cancer, prostate cancer, leukemia).

A compound that decreases the activity of a SAP family member protein (*e.g.*, by decreasing SAP family member protein gene expression or biological activity) may be used to decrease the ability of a SAP family member protein to modulate SH2 domain-containing protein mediated signal transduction. For example, a compound that decreases a SAP family member protein, thereby decreasing antigen-specific T

cell activation, would be advantageous in the treatment of diseases involving an excessive level of antigen-specific T cell activation, such as autoimmune diseases.

Preferred screens for compounds that affect a SAP family member protein's modulation of SH2 domain-containing protein-mediated signal transduction are rapid and high through-put. Using as an example the ability of SAP protein to enhance antigen-specific T cell activation, syngeneic antigen-presenting cells (APCs) expressing, for example, an ovalbumin peptide, may be cultured in a multiwell (*e.g.*, a 96 well microtiter) plate. T cells that specifically recognize ovalbumin peptide in context with the MHC expressed on the cultured syngeneic antigen presenting cells may be transfected with DNA that includes the promoter of an gene whose expression is upregulated upon antigen-specific T cell activation (*e.g.*, the promoter from the gene encoding IL-2) operably linked to a reporter gene, such as green fluorescent protein (GFP). The T cells are added to each peptide plus APC-containing well, followed by addition to each well of a compound, or combination thereof, being screened for an ability to modulate SAP protein-mediated enhancement of antigen-specific T cell activation. The compound-treated plate is then subjected to analysis on a 96 well fluorescent plate reader for the presence of GFP. A well with increased GFP expression compared to a control well not treated with any compound indicates a compound with an ability to modulate SAP protein-mediated enhancement of antigen-specific T cell activation.

Molecules that are found, by the methods described above, to effectively modulate gene expression of a SAP family member protein or biological activity of such a protein may be tested further in animal models. If they continue to function successfully in an *in vivo* setting, they may be used as therapeutics to either inhibit or enhance SH2 domain-containing protein-mediated signal transduction, as appropriate.

c) Screens for Additional Reagents which Mimic SAP Family Member Proteins

Different compounds may have the same mechanism of action without concomitant similarity in size and/or structure. Accordingly, although peptide and

non-peptide mimetics based upon the SH2 domain of a SAP family member protein are likely to induce an SAP family member-like modulation of SH2 domain-containing protein-mediated signal transduction in cells having such a signal transduction pathway, non-structural mimetic reagents may also have this capability. Utilizing the SH2 domain-containing protein-mediated signal transduction assays described herein, such reagents may be identified.

d) Identification and Generation of SAP Family Member Protein SH2 Domain Peptide Mimetics

One efficient method to treat patients (suffering from, for example, XLP disease) with the SH2 domain of a SAP family member is to generate mimetics which possess the non-phosphorylated tyrosine residue-binding abilities of the SAP family member proteins. Peptide mimetics of the SH2 domain of a SAP family member, or DNA encoding these peptides, may be used in concert. For example, two or more different peptides which correspond to different regions of the SAP protein SH2 domain may be introduced into the same population of cells. Likewise, a peptide that corresponds to the SH2 domain of an SAP protein may be introduced with a peptide that corresponds to the SH2 domain of an EAT-2 protein.

Synthetic peptides corresponding to the SH2 domain or a SAP family member protein may purchased from commercially available sources (such as the peptide generating facility at the Department of Biochemistry and Molecular Pharmacology at Harvard Medical School, Boston, MA), and tested for an ability to affect SH2 domain-containing protein mediated signal transduction events, such as the ability of a SAP family member to inhibit antigen-specific T cell activation, in various signal transduction detecting assays known in the art and described herein. Such peptides may be taken up directly by cells in culture or delivered to the cells by a variety of methods, including lipid vesicles or electroporation. In addition, nucleic acid sequences encoding these peptides may be subcloned into the cloning site of an expression cloning vector and the plasmid DNA introduced to the cell of interest by various transfection methods known in the art (*e.g.*, electroporation, DEAE-dextran,

calcium phosphate). DNA encoding peptides corresponding to the SH2 domain of a SAP family member may also be incorporated into coding sequences of fusion proteins and the mimetic delivered by transfection of the fusion protein encoding expression vector or fusion protein encoding viral vectors.

Peptides, or combinations thereof, may be screened for efficacy and effective dose requirements using the various SH2 domain-containing protein-mediated signal transduction assays well known in the art. Such SH2 domain-containing protein-mediated signal transduction assays include *in vitro* kinase and *in vitro* phosphatase assays of SH2 domain-containing or phosphotyrosine residue-containing proteins with kinase and phosphatase activities, respectively. In another example, various concentrations of peptide may be introduced with the SAP family member promoter-luciferase plasmid described herein.

DNA encoding potential peptide mimetics of the SH2 domain of a SAP family member may also be identified by hybridization of the DNA to the nucleotide sequences encoding SAP protein (SEQ ID NOs: 3 and 5) provided on Figs. 2A and 2C, or to the nucleotide sequences encoding EAT-2 protein (GenBank Accession Nos. AF020263 and AF020264). In one particular example of this approach, DNAs may be identified by an ability to hybridize to the SAP family member-encoding nucleotide sequences under high stringency conditions. High stringency conditions may include hybridization at about 40°C in about 2XSSC and 1%SDS, followed by a first wash at about 65°C in about 2XSSC and 1%SDS, and a second wash at about 65°C in about 1XSSC. Other hybridization stringency conditions, both high and low, are defined in the art (see, for example, Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2nd ed.), CSH Press, 1989, or Ausubel, *et al.*, *supra*).

Once identified, a DNA which hybridizes to the nucleotide sequences encoding a SAP family member may used to generate a polypeptide product by standard techniques. For example, the DNA may be subcloned into the pCDNA3.1 expression plasmid (commercially available from Clontech) and the resulting plasmid

transfected into, for example, COS cells (commercially available from the ATCC), to produce recombinant polypeptide. This polypeptide product may then be screened for SAP biological activity using the various SH2 domain-containing protein-mediated signal transduction assays described herein.

The identification of minimal peptide killing sequences allows the generation of non-peptidic mimetics. Techniques for generating such non-peptidic mimetics of the SH2 domain of a SAP family member are standard chemistry techniques and well known to one skilled in the art of combinatorial chemistry. The efficacies of these non-peptide mimetics may be assayed similarly to peptide mimetics of the SAP family member SH2 domain.

VI. Therapies

Therapies may be designed to circumvent or overcome a SAP gene defect or inadequate SAP family member gene expression, and thus modulate and possibly alleviate conditions involving an inappropriate amount of SH2 domain-containing protein-mediated signal transduction. Hence, in considering various therapies, such therapies may be targeted at any tissues demonstrated to express SAP family member protein (*e.g.*, SAP protein in T cells). With particular focus on the immune system, therapies to inhibit human SAP family member gene expression are useful in reducing antigen-specific T cell activation in autoreactive T lymphocytes. Likewise, therapies to enhance SAP family member gene expression are useful in enhancing antigen-specific T cell activation in, for example, XLP patients.

SH2 domain-containing protein-mediated signal transduction-modulating SAP family member reagents may include, without limitation, full length or fragment SAP family member polypeptides, SAP family member antisense nucleic acid, or any compound which increases a SAP family member's SH2 domain-containing protein-mediated signal transduction-modulating activity.

a) Protein Therapy

Treatment or prevention of inappropriate SH2 domain-containing protein-

mediated signal transduction can be accomplished by replacing mutant or surplus SAP family member protein with normal protein, by modulating the function of mutant protein, or by delivering normal SAP family member protein to the appropriate cells. It is also be possible to modify the pathophysiologic pathway (*e.g.*, a signal transduction pathway involving SH2 domain containing proteins) in which the SAP protein participates in order to correct the physiological defect.

To replace a mutant protein with normal protein, or to add protein to cells which no longer express sufficient levels of a SAP family member, it is necessary to obtain large amounts of pure SAP family member protein from cultured cell systems which can express the protein. Delivery of the SAP family member protein to the affected cells can then be accomplished using appropriate packaging or administrating systems. Alternatively, small molecule analogs may be used and administered to act as SAP family member protein agonists and in this manner produce a desired physiological effect. Methods for finding such molecules are provided herein.

b) Gene Therapy

Gene therapy is another potential therapeutic approach in which normal copies of the SAP family member encoding gene are introduced into T cells to successfully encode for normal and abundant protein in cells which undergo inappropriately low levels of T cell activation. Likewise, nucleic acids encoding SAP family member antisense RNA may be introduced into T cells to inhibit an inappropriately high level of T cell activation. SAP family member-encoding sequences or SAP family member antisense RNA may also be introduced into non-lymphoid cells, to modulate inappropriate levels of SH2 domain-containing protein-mediated signal transduction. The SAP family member-encoding sequence (or antisense SAP family member RNA-encoding sequence) must be delivered to those cells in a form in which it can be taken up and encode for sufficient protein to provide effective function. Preferably, such sequences are operably linked to the endogenous SAP family member gene promoter. Alternatively, in some mutants it may be possible to promote normal levels of SH2 domain-containing protein-mediated signal transduction by introducing another copy

of the homologous gene bearing a second mutation in that gene or to alter the mutation, or use another gene to block any negative effect.

Transducing retroviral vectors can be used for somatic cell gene therapy especially because of their high efficiency of infection and stable integration and expression. The targeted cells however must be able to divide and the expression levels of normal protein should be high. For example, the full length SAP family member gene, or portions thereof, can be cloned into a retroviral vector and driven from the endogenous SAP family member gene promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest (*e.g.*, the CD2 promoter in T cells). Other viral vectors which can be used include adenovirus, adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpes virus such as Epstein-Barr Virus.

Retroviral vectors, adenoviral vectors, adenovirus-associated viral vectors, or other viral vectors with the appropriate tropism for cells likely to be involved in SH2 domain-containing protein-mediated signal transduction (for example, T cells) may be used as a gene transfer delivery system for a therapeutic SAP family member gene construct. Numerous vectors useful for this purpose are generally known (Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis and Anderson, BioTechniques 6: 608-614, 1988; Tolstoshev and Anderson, Curr. Opin. Biotech. 1: 55-61, 1990; Sharp, The Lancet 337: 1277-1278, 1991; Cornetta *et al.*, Nucl. Acid Res. and Mol. Biol. 36: 311-322, 1987; Anderson, Science 226: 401-409, 1984; Moen, Blood Cells 17: 407-416, 1991; Miller *et al.*, Biotech. 7: 980-990, 1989; Le Gal La Salle *et al.*, Science 259: 988-990, 1993; and Johnson, Chest 107: 77S-83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg *et al.*, N. Engl. J. Med 323: 370, 1990; Anderson *et al.*, U.S. Patent No. 5,399,346).

Gene transfer could also be achieved using non-viral means requiring transfection *in vitro*. Transplantation of normal genes into the affected cells of a patient can also be useful therapy. In this procedure, a normal SAP family member

gene is transferred into a cultivatable cell type, either exogenous or endogenous to the patient. These cells are then injected serotologically into the targeted tissue(s). In addition, SAP family member-encoding DNA may be introduced into a T cell by lipofection (Felgner *et al.*, Proc. Natl. Acad. Sci. USA 84: 7413, 1987; Ono *et al.*, Neurosci. Lett. 117: 259, 1990; Brigham *et al.*, Am. J. Med. Sci. 298: 278, 1989; Staubinger *et al.*, Meth. Enz. 101: 512, 1983), asialorosonucoid-polylysine conjugation (Wu *et al.*, J. Biol. Chem. 263: 14621, 1988; Wu *et al.*, J. Biol. Chem. 264: 16985, 1989); or, less preferably, micro-injection under surgical conditions (Wolff *et al.*, Science 247: 1465, 1990).

In another approach that may be utilized with all of the above methods, a therapeutic SAP family member DNA construct is preferably applied to the site of the desired normal SH2 domain-containing protein-mediated signal transduction (for example, by injection). However, it may also be applied to tissue in the vicinity of the affected SH2 domain-containing protein-mediated signal transduction event or to a blood vessel supplying the cells with an inappropriate level of SH2 domain-containing protein-mediated signal transduction.

In the constructs described, SAP family member cDNA expression can be directed from any suitable promoter (*e.g.*, the human cytomegalovirus (CMV), simian virus 40 (SV40), or, preferably, the endogenous SAP gene promoter), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in neural cells, lymphocytes, or muscle cells may be used to direct expression of a SAP family member protein. The enhancers used could include, without limitation, those that are characterized as tissue- or cell-specific in their expression. Alternatively, if a SAP family member genomic clone is used as a therapeutic construct, regulation may be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

Antisense based strategies may be employed to explore SAP family member

gene function and as a basis for therapeutic drug design. Antisense strategies may use a variety of approaches, including the use of antisense oligonucleotides and injection of antisense RNA. For our analysis of SAP family member gene function, a method of transfection of antisense RNA expression vectors into targeted cells is employed. Phenotypic effects induced by antisense effects are based on changes in criteria such as protein levels, protein activity measurement, and target mRNA levels.

For example, SAP family member gene therapy may also be accomplished by direct administration of antisense SAP family member mRNA to a cell that is expected to undergo an undesirably high level of T cell activation (*e.g.*, an autoreactive T cell). The antisense SAP family member mRNA may be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using an antisense SAP family member cDNA under the control of a high efficiency promoter (*e.g.*, the T7 promoter). Administration of antisense SAP family member nucleic acid to cells can be carried out by any of the methods for direct nucleic acid administration described above.

Another therapeutic approach within the invention involves administration of recombinant SAP polypeptide, either directly to the site of a desired inhibition of SH2 domain-containing protein-mediated signal transduction event (for example, by injection) or systemically (for example, by any conventional recombinant protein administration technique). The dosage of SAP family member depends on a number of factors, including the size and health of the individual patient, but, generally, between O.l mg and 100 mg inclusive are administered per day to an adult in any pharmaceutically acceptable formulation.

c) Preventative Therapies

In a patient diagnosed to have a SAP family member mutation or to be susceptible to SAP family member mutations or to have aberrant expression of a SAP family member (even if those mutations or expression patterns do not yet result in underexpression or decreased biological activity of a SAP family member), or a patient diagnosed with a XLP disease, or diagnosed with an infectious disease (*e.g.*,

AIDS), any of the above therapies may be administered before the occurrence of the disease phenotype. For example, the therapies may be provided to an XLP patient who does not yet show EBV-infection induced non-Hodgkin's lymphoma. In particular, compounds shown to increase SAP family member expression or SAP family member biological activity may be administered to patients diagnosed with infectious diseases or cancer by any standard dosage and route of administration (see above). Alternatively, gene therapy using a antisense SAP family member mRNA expression construct may be undertaken to reverse or prevent the T cell defect prior to the development of an autoimmune disease.

The methods of the instant invention may be used to reduce or diagnose the disorders described herein in any mammal, for example, humans, domestic pets, or livestock. Where a non-human mammal is treated or diagnosed, the SAP family member polypeptide, nucleic acid, or antibody employed is preferably specific for that species.

VII. Administration of SAP Reagents and Modulators

A SAP family member protein, gene, or modulator may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer neutralizing SAP family member-specific antibodies or SAP family member-inhibiting compounds (*e.g.*, antisense SAP family member or a SAP family member dominant negative mutant) to patients suffering from a disease characterized by an excessive level of T cell activation (*e.g.*, an autoimmune disease). Likewise, a SAP family member protein, a cDNA encoding a SAP family member protein, or a mimetic thereof, may be administered to a patient suffering from a disease characterized by an insufficient level of T cell activation (*e.g.*, XLP or cancer). Administration may begin before the patient is symptomatic. Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intra-arterial, subcutaneous,

intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in Remington's Pharmaceutical Sciences, (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for SAP family member modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

If desired, treatment with a SAP family member protein, gene, or modulatory compound may be combined with more traditional therapies for the disease involving excessive or insufficient levels of SH2 domain-containing protein-mediated signal transduction.

VIII. Detection of Conditions Involving Altered SH2 Domain-Containing Protein-Mediated Signal Transduction

SAP family member polypeptides and nucleic acid sequences find diagnostic use in the detection or monitoring of conditions or diseases involving signal

transduction pathways which include SH2 domain-containing proteins. For example, increased expression of a SAP family member may be correlated with diseases hallmarked by decreased SH2 domain-containing protein-mediated signal transduction in humans. Accordingly, a decrease or increase in the level of SAP family member protein production may provide an indication of a deleterious condition. Levels of expression of SAP family member proteins may be assayed by any standard technique. For example, SAP family member expression in a biological sample (*e.g.*, a biopsy) may be monitored by standard Northern blot analysis or may be aided by PCR (see, *e.g.*, Ausubel *et al.*, *supra*; PCR Technology: Principles and Applications for DNA Amplification, H.A. Ehrlich, Ed. Stockton Press, NY; Yap *et al.*, Nucl. Acids. Res. 19: 4294, 1991).

Alternatively, a biological sample obtained from a patient may be analyzed for one or more mutations in SAP nucleic acid sequences using a mismatch detection approach. Generally, these techniques involve PCR amplification of nucleic acid from the patient sample, followed by identification of the mutation (*i.e.*, mismatch) by either altered hybridization, aberrant electrophoretic gel migration, binding or cleavage mediated by mismatch binding proteins, or direct nucleic acid sequencing. Any of these techniques may be used to facilitate mutant SAP family member detection, and each is well known in the art; examples of particular techniques are described, without limitation, in Orita *et al.* (Proc. Natl. Acad. Sci. USA 86: 2766-2770, 1989) and Sheffield *et al.* (Proc. Natl. Acad. Sci. USA 86: 232-236, 1989).

In yet another approach, immunoassays are used to detect or monitor SAP family member protein expression in a biological sample. SAP family member-specific polyclonal or monoclonal antibodies (produced as described above) may be used in any standard immunoassay format (*e.g.*, ELISA, Western blot, or RIA) to measure SAP family member polypeptide levels. These levels would be compared to wild-type SAP family member polypeptide levels. For example, an increase in SAP production may indicate a condition involving insufficient antigen-specific T cell activation. Examples of immunoassays are described, *e.g.*, in Ausubel *et al.*, *supra*.

Immunohistochemical techniques may also be utilized for SAP family member protein detection. For example, a tissue sample may be obtained from a patient, sectioned, and stained for the presence of SAP protein using an anti-SAP antibody and any standard detection system (*e.g.*, one which includes a secondary antibody conjugated to horseradish peroxidase). General guidance regarding such techniques can be found in, *e.g.*, Bancroft and Stevens (Theory and Practice of Histological Techniques, Churchill Livingstone, 1982) and Ausubel *et al.* (*supra*).

In one preferred example, a combined diagnostic method may be employed that begins with an evaluation of SAP family member protein production (for example, by immunological techniques or the protein truncation test (Hogerrorst *et al.*, Nature Genetics 10: 208-212, 1995) and also includes a nucleic acid-based detection technique designed to identify more subtle SAP family member mutations (for example, point mutations). As described above, a number of mismatch detection assays are available to those skilled in the art, and any preferred technique may be used. Mutations in SAP family member-encoding nucleic acids may be detected that either result in loss of expression of SAP family member or loss of normal SAP family member biological activity. In a variation of this combined diagnostic method, SAP family member biological activity is measured as antigen-specific T cell activation-inhibiting activity using any appropriate antigen-specific T cell activation assay system (for example, those described herein).

Mismatch detection assays also provide an opportunity to diagnose a SAP family member-mediated predisposition to diseases caused by inappropriate SH2 domain-containing protein-mediated signal transduction. For example, a patient heterozygous for a SAP family member mutation that induces a reduced level of SAP family member expression may show no clinical symptoms and yet possess a higher than normal probability of developing serious disease. Given this diagnosis, a patient may take precautions to minimize their exposure to adverse environmental factors (for example, UV exposure or chemical mutagens) and to carefully monitor their medical condition (for example, through frequent physical examinations). This type of SAP

family member diagnostic approach may also be used to detect SAP mutations in prenatal screens. The SAP family member diagnostic assays described above may be carried out using any biological sample (for example, any biopsy sample or other tissue) in which the SAP family member is normally expressed. Identification of a mutant SAP family member-encoding gene may also be assayed using these sources for test samples.

Alternatively, a SAP family member mutation, particularly as part of a diagnosis for predisposition to SAP family member-associated cancer, may be tested using a DNA sample from any cell, for example, by mismatch detection techniques. Preferably, the DNA sample is subjected to PCR amplification prior to analysis.

IX. Identification of Additional SAP Family Member Genes

Standard techniques, such as the polymerase chain reaction (PCR) and DNA hybridization, may be used to clone additional SAP family member homologues in other species. Southern blots of murine genomic DNA hybridized at low stringency with probes specific for human or murine SAP family member proteins reveal bands that correspond to SAP family members. Thus, additional SAP family member sequences may be readily identified using low stringency hybridization. Furthermore, region of high homology to a SAP family member may be used to screen databases for partial SAP family member sequences and SAP family member-specific primers may be used to clone additional SAP family member related genes by RT-PCR.

X. Construction of a Transgenic Animal

Characterization of the murine and human SAP family member genes (i.e., SAP protein encoding genes and EAT-2 protein encoding genes) provides information that is necessary for the construction of SAP family member knockout animal models by homologous recombination or by other standard techniques. Preferably, the model is a mammalian animal, most preferably a mouse. The murine SAP family member genomic DNA described herein enables the development of a mouse lacking an

endogenous SAP family member gene. Preferably, such a mouse with a SAP protein encoding gene knock-out may be used as a murine model of XLP disease.

Similarly, an animal model of SAP family member overproduction or an animal model of mutant SAP family member expression may be generated by integrating one or more SAP family member-encoding sequences into the genome operably linked to functional promoter sequences, according to standard transgenic techniques.

A replacement-type targeting vector, which would be used to create a knockout model, can be constructed using an isogenic genomic clone, for example, from a mouse strain such as 129/Sv (Stratagene Inc., LaJolla, CA). The targeting vector will be introduced into a suitably-derived line of embryonic stem (ES) cells by electroporation to generate ES cell lines that carry a profoundly truncated form of a SAP family member encoding gene. To generate chimeric founder mice, the targeted cell lines will be injected into a mouse blastula stage embryo. Heterozygous offspring will be interbred to homozygosity. Knockout mice would provide the means, *in vivo*, to screen for therapeutic compounds that modulate SH2 domain-containing protein-mediated signal transduction via a SAP family member-dependent pathway. Making such mice may require use of loxP sites due to the multiple copies of SAP family member encoding genes on the chromosome (see Sauer and Henderson, Nucleic Aids Res. 17: 147-61,1989).

EXAMPLES

The following examples are to illustrate the invention. They are not meant to limit the invention in any way.

Example I

Cloning of SAP, a protein that interacts with the cytoplasmic domain of SLAM

To elucidate the signaling mechanism of the SLAM, co-activator proteins that interacted with the cytoplasmic domain of human SLAM were identified using a yeast

two-hybrid system with the 77 amino acid human SLAM cytoplasmic domain as "bait" (for a description, in general, of the yeast two-hybrid system, see, for example, Gyuris *et al.*, Cell 75:791-803, 1993). To do this, the cDNA encoding the human SLAM4 cytoplasmic domain (the sequence of which is shown on Fig. 1; SEQ ID NO: 1) was cloned in the pGBT9 vector (commercially available from Clontech Laboratories Inc., Palo Alto, CA) and used to screen a cDNA library derived from the human T cell line KT3 in the yeast two-hybrid system. The yeast two-hybrid assay and isolation of positive clones and subsequent interaction analyses are carried out as has been previously described (see, for example, PCT Publication WO 95/28497).

Eight human cDNA clones were isolated that encoded a 128 amino acid polypeptide chain, which was termed SLAM-Associated Protein (SAP; Fig. 2A and 2B). The 5' end of human SAP was obtained by the RACE technique (using reagents commercially available from Clontech). To confirm that full length cDNAs had been obtained, cDNA clones were also isolated from a human T cell library (Jurkat). We submitted the human SAP cDNA and protein sequence to the GenBank database, and it has been assigned GenBank Accession No. AF073019 (NID No. g3695070).

Having identified human SAP, we searched the databases and found a 200 nucleotide long fragment showing sequence showing identity to human SAP. Mouse SAP cDNA (Figs. 2C and 2D) was thus cloned by the screening of a mouse (C57BL6) thymus cDNA library in the Zap Express vector using as a probe a cDNA amplified from mouse thymic mRNA by RT-PCR (reverse transcriptase PCR), using specific primers flanking the 5' and 3' of the EST sequence AA255258. We submitted the murine SAP cDNA and protein sequence to the GenBank database, and it has been assigned GenBank Accession No. AF072903 (NID No. g3695066).

The predicted SAP protein contains a single SH2 domain (residues 6-102) followed in human SAP by a short 26 amino acid tail (residues 103-128 in human SAP) and in murine SAP by a short 24 amino acid tail (residues 103-126 in murine SAP). SAP was determined to contain an SH2 domain based on the close identity of five "blocks" of sequences within the putative SH2 domain with "blocks" found in

SH2 domain-containing proteins using the position-based method of Henikoff and Henikoff, J. Mol. Biol. 243: 574-578, 1994; Henikoff and Henikoff, Genomics 19: 97-107, 1994. Shown on Fig. 4A is the human SAP amino acid sequence with the positions of the five SH2 domain-hallmarking "blocks" shown in bold. In Fig. 4B, the sequences of these blocks is shown. Each of these "blocks" has a high degree (*e.g.*, above 60%) sequence identity with a corresponding block in the SH2 domain of an SH2 domain-containing protein.

However, since SAP was isolated using a two-hybrid screen in yeast, which do not phosphorylate tyrosine residues, we do not define SAP as an SH2 domain-containing protein.

Human and murine SAP are highly homologous (96% identical) both in the SH2 and tail domains. In Fig. 5, human and murine SAP amino acid sequences were compared with the SH2-domains which were found most similar in a computer aided search, namely human SHIP, murine EAT-2 (GenBank Accession No. AF020263; Thompson *et al.*, Oncogene 13: 2649-2658, 1996), and human Abl. A computer-aided search for the SAP cDNA in the expressed-sequence tag library (EST) led to the identification of 3 human clones (Accession Nos. N89899, W19453 (Fetal Lung), and AA354319 (Jurkat)), and one highly homologous murine T cell derived clone of 294 bp (Accession No. AA255258). Whereas its SH2-domain was clearly related to other SH2-domains, particularly those of SHIP (45% identical), EAT-2 (40% identical), and Abl (35% identical), the short tail of SAP was unlike any known protein domain.

We next generated rabbit antisera containing polyclonal anti-SAP antibodies. To do this, human SAP (hSAP; CQGTTGIREDPDV) and murine SAP (mSAP; CQAPTGRRDSDI) peptides were coupled to keyhole limpet hemocyanin (KLH; commercially available from Pierce Chemical Co., Rockford, IL) and used to inject New Zealand rabbits. Rabbits antisera were obtained using standard methods (see, for example, Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1994).

The anti-human SAP antibodies were used to prepared immunoprecipitates of

whole lysates prepared from human T cell tumor cells (Jurkat) or human peripheral blood leukocytes (PBL) (Fig. 6A). Likewise, the anti-murine SAP antibodies were used to prepare immunoprecipitates of whole lysates prepared from murine (C57BL/6) thymocytes (Fig. 6B). The crude lysate was first precleared using 50 µl of protein G-agarose beads (GIBCO BRL) and 5 µl of normal mouse serum or normal rabbit serum for 1 hour. Immunoprecipitations were carried out using the indicated antibodies and 30 µl of protein G-agarose beads for 3 hours at 4°C. Beads were then washed as described before (Ramesh *et al.*, Proc. Natl. Acad. Sci. USA 94: 14671-14676, 1997), and bead-associated proteins resolved by SDS-PAGE. The SDS-PAGE resolved proteins were transferred onto PVDF filters (commercially available from Millipore Corp., Bedford, MA), which were then blocked for 1 hour with 5% Skim-milk (or 3% Bovine Serum Albumin). Following Western blotting analysis with anti-human SAP (Fig. 6A) or anti-murine SAP (Fig. 6B), bound antibody was revealed using horseradish peroxide-conjugated secondary antibodies using enhanced chemiluminescence (Supersignal; commercially available from Pierce Chemical Co.).

Antibodies directed at human SAP tail sequences detected a 15 kD protein in anti-SAP immunoprecipitates of detergent cell lysates made either from a human T cell tumor (Jurkat) or human peripheral blood leukocytes (PBL) (Fig. 6A). Likewise, antibodies directed at murine SAP tail sequences detected a 15 kD protein in detergent lysates made from murine thymocytes (Fig. 6B). Because the observed molecular weight was consistent with the predicted molecular mass, the human and mouse SAP cDNAs are unlikely to encode a fragment of a larger protein.

We next generated reagents to determine if SLAM and SAP interacted. cDNA encoding the entire coding region of human SAP (SEQ ID NO: 3) was next inserted into the GST fusion protein expression vector pGEX2T (commercially available from Pharmacia Biotech, Uppsala, Sweden) to generate the GST-SAP construct. The GST-SAP fusion protein-encoding plasmid was then used to transform *E. coli* bacteria, and, after propagation of the transformed bacteria, GST-SAP fusion

proteins were purified from lysed bacteria according to standard protocols. The SLAM construct was generated subcloning human SLAM4 cDNA in vector pJFE14-SRα. El-4 cells were stably transfected by electroporation with a cDNA encoding SLAM4 or vector alone. Cells then were selected by growth in media with 600 mg/ml neomycin for three weeks. Cells then were stained with anti-SLAM PE conjugated antibodies (A12) and positive cells were sorted.

Following cell surface biotinylation (using the EZ-Link Sulfo-NHS-Biotin reagent according to manufacturer's instructions; Pierce Chemical Co.) of untransfected EL-4 cells and SLAM transfected EL4 cells, whole lysates were prepared and incubated for 3 hours with 5 μg of GST or SAP-GST fusion protein in the presence of glutathione beads. Following incubation, the beads were washed three times, resolved by SDS-PAGE, and transferred onto PVDF filters, which were then blocked for 1 hour with 5% Skim-milk (or 3% Bovine Serum Albumin). In Fig. 6C, Western blotting analysis was performed using streptavidin-HRP (horseradish peroxide-conjugated streptavidin, which specifically binds biotinylated proteins).

SLAM and SAP interact in T lymphocytes, as shown by the ability of a human SAP-GST fusion protein to specifically precipitate SLAM from detergent lysates made from a murine T cell (EL-4) transfected with human SLAM (Fig. 6C). In contrast, no interaction was detected in lysates made with untransfected EL4 cells (Fig. 6C). SAP, as detected by Western blotting with anti-SAP antibody, was also co-immunoprecipitated with anti-SLAM antibodies in detergent lysates prepared from PHA-activated human peripheral blood lymphocytes (as described in Isomaki *et al.*, J. Immunol. 159: 2986-2993, 1997) (top panel, Fig. 6D). Immunoprecipitations with anti-SLAM antibodies were performed as described above for anti-SAP immunoprecipitation.

Example II

Identification of SAP as a T cell protein

We next did a series on Northern blotting analyses with SAP probes to

determine the tissue distribution of SAP mRNA. poly(A+) RNA from various human tissues (Clontech) or from total mRNA obtained from cell lines, according to standard techniques and using the Trizol reagent (commercially available from GIBCO BRL). Northern blotting analysis was performed (see, for example, Ausubel *et al.*, *supra*), using a radiolabelled human SAP-specific cDNA as a probe.

The level of human SAP mRNA expression was found to be highest in the thymus and lower in spleen and peripheral blood lymphocytes (Fig. 7A). SAP was expressed in all major subsets of human T cells (CD4+, CD45RO+, CD45RA+, and CD8+) (Fig. 7C) and in the T cell tumor, Jurkat, and the EBV+ Burkitt lymphoma line, Raji. No transcripts were detected in the EBV+ Burkitt lymphomas, Namalwa and BL41/B95-8; the EBV transformed cell line, X50-7; the EBV+ marmoset cell lines, B95-8 and FF41; the EBV- Burkitt lymphoma, BJAB; or the pre-B ALL-lines, LAZ 221 and NALM 6 (Figs. 7B and 7C, and data not shown). The low level of SAP mRNA that was detected in the small intestine probably resulted from lymphocyte contamination. In *wt* mice, SAP is expressed in T cells, but not in B cells, while no SAP mRNA or protein could be detected in $CD3\epsilon^{Null}$ mice which lack T cells. Both human mRNA species encoding SAP (2.5 and 0.9 kb) were represented amongst the cDNA clones isolated in the two-hybrid system and encoded the same open reading frame, but differed in their 3' untranslated sequences. Collectively, these studies showed that, unlike SLAM, SAP was primarily expressed in T cells.

Example III

SAP is encoded by the X-linked Lymphoproliferative disease (XLP) gene

Using a 45 kb pBAC clone that contained all four exons of murine SAP (see Fig. 5), the SAP gene was localized within band A5.1 of the murine X chromosome. Because of synteny between murine band A5.1 and human Xq25, the locus at which the immune deficiency X-linked Lymphoproliferative disease (XLP) had been mapped (Lamartine *et al.*, Eur. J. Hum. Genet. 4: 342-351, 1996; Porta *et al.*, Genome Research. 7: 27-36, 1997; Lanyi *et al.*, *supra*; Purtilo, D.T., *supra*), it was plausible

that the SAP gene was involved in this disease. Moreover, the observation that uncontrolled B cell proliferation following EBV infection in XLP patients often leads to fatal infectious mononucleosis, malignant lymphomas, hypogammaglobinemia, or aplastic anemia (Purtilo, D.T., *supra*; Seemayer *et al.*, Pediatr. Res. 38: 471-478, 1995) was in agreement with the involvement of SLAM in T↔B cell interactions. To test the hypothesis that SAP was encoded by the XLP gene, SAP cDNAs were isolated from peripheral blood mononuclear cells of three XLP patients: a previously described patient A1 (Rousset *et al.*, Clin. Exp. Immunol. 63: 280-289, 1986), and a set of two brothers, B1 and B2, who were recently diagnosed with XLP.

XLP patient A1 developed hypogammaglobunemia and recurrent pulmonary infections a few months after severe EBV induced infectious mononucleosis (Rousset *et al.*, *supra*). His family history and the persistence of an unbalanced virus-host relationship after EBV infection are consistent with the main characteristics of XLP syndrome (Rousset *et al.*, *supra*). We used RT-PCR (reverse transcriptase-PCR) to elucidate whether or not patient A1 had a mutation in the SAP gene. RT-PCR was performed using GenAmp RT-PCR kit (Perkin Elmer Corp., Norwalk, CT) using the following primers: forward primer 5'-GCC TGG CTG CAG TAGCAG CGG CAT CTC CC-3' (SEQ ID NO: 17); and reverse primer 5'-ATG TAC AAA AGT CCA TTT CAG CTT TGA C-3' (SEQ ID NO: 18). The RT-PCR products were then subjected to 10% polyacrylamide gel electrophoresis at 200 volts for 7 hr. The gels were stained with CYBR Green (Molecular Probes Inc., Eugene, OR) for 20 min., according to manufacturer's procedures, and visualized and photographed using a UV illuminator.

When RT-PCR products of T cells from patient A1 were analyzed by polyacrylamide gel electrophoresis (Fig. 8A), initially three products were found with the sizes: 629 bp (the full length SAP coding sequence); 565 bp; and 520 bp. Upon cloning of the RT-PCR products of patient A1 four different mRNAs were identified: (i) full length hSAP; (ii) a coding sequence, ΔE2, lacking the 64 nucleotides of exon 2 (A1-1 in Figs. 3, 9A, and 9B); (iii) E3Δ55 with a 55 nucleotide deletion in exon 3

(hSAPΔ55 in Figs. 3, 9A, and 9B); and (iv) a cDNA with both a deleted exon 2 and the deletion E3Δ55 (A1-2 in Figs. 3, 9A, and 9B). Thus, T cells from patient A1 had four mRNA species coding for different forms of SAP, two of which were detected in healthy individuals (629 and 574 bp) and two that were specific for the patient (A1-1 of 565 bp and A1-2 of 520 bp). (Note that the 574 and 565 bp species cannot be separated on PAGE in Fig. 8A.) The predicted amino acid sequences suggested two truncated proteins in which essentially only the first exon of SAP was intact.

RT PCR products from peripheral blood mononuclear cells of 60 healthy individuals contained two mRNA species (629 and 574 bp), as judged by polyacrylamide gel electrophoresis and nucleotide sequence analyses (Figs. 8A and 9B). These represented full length hSAP and E3Δ55 (hSAPΔ55 in Figs. 3, 9A and 9B), in which part of exon 3 had been deleted. The E3Δ55 coding sequence started at the beginning of exon 3 at nucleotide position 288 and ended at nucleotide 342 (Fig. 9A). Because of a frame shift, the predicted protein sequence was identical to that of the first two exons of SAP (amino acids 1-67) followed by a nine amino acid sequence and a stop codon (Fig. 9B). Taken together, the mRNAs of patient A1 either had a deletion of exon 2, or a deletion of exon 2 in addition to a deletion of exon3, which was a normal variant. These mutant forms derived from patient A1 were estimated to represent 90% of the patient's SAP mRNA.

To aid our analysis, each of the four exons of human SAP gene was amplified by PCR using the following primers:

forward primer 5'-GCC CTA CGT AGT GGG TCC ACA TAC CAA CAG-3' (SEQ ID NO: 19), and reverse primer 5'-GCA GGA GGC CCA GGG AAT GAA ATC CCC AGC-3' (SEQ ID NO: 20) for exon 1;

forward primer 5'-GGA AAC TGT GGT TGG GCA GAT ACA ATA TGG-3' (SEQ ID NO: 21), and reverse primer 5'-GGC TAA ACA GGA CTG GGA CCA AAA TTC TC-3' (SEQ ID NO: 22) for exon 2;

forward primer 5'-GCT CCT CTT GCA GGG AAA TTC AGC CAA CC-3' (SEQ ID NO: 23), and reverse primer 5'-GCT ACC TCT CAT TTG ACT TGC TGG CTA

CAT C-3' (SEQ ID NO: 24) for exon 2; and

forward primer 5'-gac agg gac cta ggc tca ggc ata aac tga c-3' (SEQ ID NO: 25), and reverse primer 5'- ATG TAC AAA AGT CCA TTT CAG CTT TGA C -3' (SEQ ID NO: 26) for exon 4. These PCR primers were designed based on the genomic sequence of human SAP released by the EMBL database (Accession No. AL022718). The PCR products were visualized on 2% agarose gels, and subcloned into pCR2.1 vector (commercially available from Invitrogen Corp., Carlsbad, CA followed by sequencing.

The 55 nucleotide deletion in exon 3 in healthy individuals was readily explained by a infrequently used splicing acceptor site within that exon: CAG preceded by a CT rich stretch of nucleotides (Lewin, B., Genes V, pp. 1-1272, Oxford University Press, Oxford, 1994) (see Fig. 10A). However, in order to explain the frequent deletion of exon 2 in patient A1, the intron sequences surrounding exon 1, 2, and 3 needed to be determined. To this end, a PCR assay based upon sequences of at least 150 nucleotides of all four introns was designed. This was facilitated by a very recent sequence in the EMBL database (Accession No. AL022718) stating that the XLP gene encoded a protein, termed DSHP. Its cDNA, genomic DNA, and protein sequences were identical to human SAP and, in addition, the exon/intron boundaries reported were identical to those determined for the mouse gene (see Fig. 5). Upon sequencing of the 443 bp PCR product derived from the exon 2 area of patient A1's DNA, a C→G mutation was found in the nucleotide adjacent to the exon 2 splice acceptor site in the DNA of patient A1 (Fig. 10A). This mutation in other gene sequences is known to affect the efficiency of the splice acceptor site (Lewin, B., *supra*) and explained the skipping of exon 2 in the majority (~ 90%) of the patient's SAP mRNA species.

To exclude the possibility that the point mutation found in XLP patient A1 represented a genetic polymorphism, a restriction enzyme assay was designed. This assay was based on the fact that the C→G mutation generated a novel Mnl I restriction enzyme recognition site in the 443 bp exon 2 PCR product. For the analysis of a

point mutation in the region preceding exon 2, exon 2 PCR products were gel-purified (using reagents commercially available from Qiagen Inc., Santa Clarita, CA) and digested with restriction enzyme Mnl I (commercially available from New England Biolabs Inc., Beverly, MA), followed by 10% polyacrylamide gel electrophoresis at 200 volts for 3 hr. As shown in Fig. 10B, digestion with Mnl I generated four fragments (187, 151, 64, and 41 bp) with A1's 443 bp PCR product, while DNA samples from 108 healthy individuals generated three fragments (251, 151, and 41 bp) (see, *e.g.*, CT-1, CT-2, and B3 in Fig. 10B). Because patient A1 was Italian, DNA from 50 healthy Italian females was analyzed as controls (providing 100 X-chromosomes). An additional 86 X-chromosomes were from 28 female (*i.e.*, 56 X-chromosomes) and 30 male (*i.e.*, 30 X-chromosomes) individuals with a random genetic background from area of the Boston, Massachusetts. Collectively, the data demonstrate that the XLP patient A1 had a mutation affecting the exon 2 splice acceptor site, which gave rise to severely truncated forms of SAP. Since this mutation was not detected in 186 X-chromosomes from healthy individuals, it did not represent a genetic polymorphism.

Two additional XLP patients, brothers B1 and B2, had a deletion of the SAP gene, while a healthy brother B3 had a normal SAP gene. The first patient, B1, was a 23 year old male with a history of recurrent pulmonary infections, dysgammaglobulinemia (elevated IgA and IgM), poor specific antibody responses to tetanus toxoid antigen, and depressed T cell proliferative response to mitogens. He developed fever, pneumonia, and hilar adenopathy, which quickly progressed to fulminant infectious mononucleosis with hemophagocytosis. EBV infection was documented by PCR and serology. One of B1's male siblings, B2, also suffered from the XLP syndrome, which manifests itself as pancytopenia, splenomegaly, dysgammaglobulinemia, and depressed T cell proliferative responses to mitogens. A third brother, B3, appears healthy, and two additional brothers, of whom no material was available, had classical XLP symptoms: one had recurrent EBV+ non-Hodgkin's lymphomas, and another died with the diagnosis of Wegener's granulomatosis and

pulmonary infiltrates.

In contrast to the healthy sibling B3, no RT-PCR product encoding hSAP could be isolated from the peripheral blood mononuclear cells of patients B1 and B2 (Fig. 8A). This was because no mRNA could be detected in Northern blotting experiments with RNA from patients B1 (not shown) and B2 (Fig. 8B). This result suggested that either the SAP gene was not transcribed in T cells from B1 and B2, or that the gene was deleted. Since none of the four exons encoding SAP could be generated in a PCR analysis based upon genomic DNA from B1 and B2 (Fig. 10C), and because a control PCR reaction for exon 2 of BRCA1 gave the expected product, we conclude that the SAP gene was deleted in XLP patients B1 and B2. By contrast, DNA from the healthy brother B3 generated PCR fragments of the expected size and with the wt sequence. Taken together, the analyses of XLP patients A1, B1, and B2 demonstrated the XLP gene encodes SAP.

Example IV

The SH2-domain of SAP binds to a specific sequence in the cytoplasmic domain of SLAM

Identification of SAP as the gene product altered in XLP raised the question of how this SLAM interactive T cell protein could account for the immunologic disturbances associated with disease. The SLAM cytoplasmic domain contains three Tyr residues (Y281, Y307, and Y327) that are surrounded by consensus SH2-domain-binding-sequences (Cocks *et al.*, *supra*), suggesting the possibility of an interaction with the SH2-domain of SAP. However, SAP was found to bound to SLAM in a yeast two-hybrid system, a system in which tyrosine residues are not phosphorylated. Hence, the SAP-SH2 domain had to bind to SLAM in a more complex fashion than through a classical interaction with a short phospho-tyrosine containing peptide.

In order to determine the portion of the cytoplasmic domain of SLAM to which the SAP SH2-domain bound, constructs encoding the CD8 extracellular and transmembrane domains, and truncated cytoplasmic domains of SLAM were

generated, and schematic diagrams of which are shown on Fig. 11A. Shown in Fig. 11B are the amino acid sequences of the cytoplasmic domains of SLAM3 and SLAM4 that were fused to CD8 extraceullar and transmembrane domains. In addition, a truncation of the cytoplasmic domain of SLAM4, the sequence of which is shown in Fig. 11B (SLAM 4 del 1), was generated by PCR from a full-length SLAM4 template, and the PCR product used to generate the CD8-SLAM del 1 fusion protein. The CD8-SLAM constructs were cloned in pCDNA3 (commercially available from Invitrogen Corp.). Human SAP cDNA was cloned in expression vector pCMV2-FLAG (commercially available from KODAK), thus generating a Flag-tagged version of human SAP. The CD8-SLAM constructs were co-expressed with Flag-tagged human SAP in COS-7 cells by transforming the cells using the DEAE-dextran method (Ausubel *et al.*, *supra*).

Following cell surface biotinylation using the EZ-Link Sulfo-NHS-Biotin reagent according to manufacturer's instructions (Pierce Chemical Co.), cell lysates which were prepared, clarified by centrifugation at 14,000 x g for 15 min. at 4°C, and the crude lysate precleared using 50 µl of protein G-agarose beads (GIBCO BRL, Gaithersburg, MD) and 5 µl of normal mouse serum or normal rabbit serum for 1 hour. Anti-CD8 (OKT3) and anti-SLAM immunoprecipitations were next performed using the antibodies and 30 µl of protein G-agarose beads for 3 hours at 4°C. The beads were then washed as described (Ramesh *et al.*, *supra*). Crude lysates and immunoprecipitates were resolved by SDS-PAGE, and transferred onto PVDF filters, which were then blocked for 1 hour with 5% Skim-milk (or 3% Bovine Serum Albumin), and then probed with anti-SAP antibodies (top panel, Fig. 12A) or streptavidin (bottom panel, Fig. 12A). Bound antibody was revealed using horseradish peroxide-conjugated secondary antibodies using enhanced chemiluminescence (Supersignal; commercially available from Pierce Chemical Co.).

Both CD8-SLAM3 (derived from a natural variant of SLAM having a short cytoplasmic domain containing only Y281) and CD8-SLAM del1 (containing Y281 and Y307), were able to co-precipitate SAP with the same efficiency as SLAM itself

(Fig. 12A). In contrast, two control chimeric proteins comprising the CD8 extracellular and transmembrane domains, and either the CD3-ϵ or the CD3-ζ cytoplasmic domain, did not co-precipitate SAP (Fig. 12A). This suggested the presence of a specific SAP binding site around the most membrane proximal tyrosine residue (Y281) of the SLAM cytoplasmic tail.

We next generated the following peptides:
SLAM Y1: CVEKKSLTIYAQVQK (SEQ ID NO: 27); SLAM pY1: CVEKKSLTI**p**YAQVQK (SEQ ID NO: 28); SLAM F1: CVEKKSLTIFAQVQK (SEQ ID NO: 29); SLAM Y2: CTTIYVAATEPVPESVQE (SEQ ID NO: 30); and SLAM Y3: CTVYASVTLPES (SEQ ID NO: 31). (Note: Rabbit polyclonal antibodies directed toward SLAM Y2 and SLAM Y3 peptides were generated as described above.) Following synthesis, the peptides were coupled to beads (sulfolink coupling gel, Pierce Chemical Co.). Coupled peptides were incubated for 1 hour with lysates from mouse thymocytes or from CD8SLAM3 transiently transfected cells. Where indicated in Figs. 12B and 12C, the coupled peptides were incubated with lysate in the presence of different concentrations of soluble peptides. Following incubation, the beads were washed three times and bead-bound proteins resolved by SDS-PAGE.

As predicted from the *in vivo* experiments, only a peptide (SLAM Y1) containing Y281, coupled to agarose beads, was able to specifically precipitate SAP from murine thymocyte lysates, but not peptides containing the other tyrosines of the cytoplasmic domain of SLAM (Fig. 12B). More importantly, binding of SAP to the agarose-coupled SLAM Y1 was blocked only by the same peptide regardless whether Y281 was replaced by Phe (SLAM F1) or by a peptide in which phospho-Tyr replaced Y281 (SLAM pY1) (Fig. 12C). This confined the binding site to a short amino acid sequence in SLAM and confirmed that phosphorylation of Y281 was not required for interactions between the two polypeptide chains. However, the SH2-domain of SAP was functionally intact (*i.e.*, it could still bind phosphorylated tyrosine residues) since it could be purified on a Sepharose-phospho-Tyr column (data not

shown).

Human SAP cDNA and mutant SAP cDNAs, generated by PCR (SAP del1, SAP del2, SAP del3, SAP del4; see Fig. 5), were next cloned in expression vector pCMV2-FLAG (commercially available from KODAK). These four SAP mutants with deletions of the SAP-tail, when co-transfected into COS-7 cells with the CD8-SLAM3 chimera, were able to interact with the short cytoplasmic domain of SLAM3, which contained the Y281 segment, as determined following anti-CD8 immunoprecipitation and Western blotting analysis with anti-FLAG antibody (Fig. 12D). Collectively these data demonstrated conclusively that SLAM interacted with SAP via part of its SH2-domain.

Example V

SAP blocks recruitment of the tyrosine phosphatase SHP-2 to the phosphorylated cytoplasmic domain of SLAM

The novelty of SLAM-SAP recognition, wherein the usual requirements for SH2-domain interactions appeared to be altered, raised the possibility that SAP was blocking physiological relevant interactions in the function of SLAM, thus acting as a natural inhibitor molecule. This model also predicted that SAP mutants that were unable to bind to SLAM would not act as inhibitors. This hypothesis was testable, because we have observed that upon phosphorylation by *c-fyn*, SLAM did recruit the tyrosine-phosphatase SHP-2.

First, two SAP mutants were expressed in COS cells: the A1 deletion (Δexon2+E3Δ55) and a classical mutation in the SH2 domain which affects binding of phosphotyrosine, SAP 32R→Q (*i.e.*, the R residue at position 32 changed to a Q residue). Neither mutant bound to the cytoplasmic tail of SLAM as judged by *in vivo* (Fig. 13A) or *in vitro* (Fig. 13B) assays. Thus, neither SAP-mutant could act as a dominant negative mutant via SLAM. More importantly, the experiment with mutant SAP 32R→Q showed that the interaction of SAP with SLAM depended upon an intact phospho-tyrosine binding pocket. Interestingly, an XLP patient with a mutation in

SAP R32 has recently been found. Taken together, the absence of binding of the two SAP mutants found in patients further supports the model that absence of SAP/SLAM interactions are critical for this disease.

Next, SLAM and *c-fyn* were co-transfected with or without SAP into COS-7 cells, which contain endogenous SHP-2. If SLAM was phosphorylated by *c-fyn*, SHP-2 was co-precipitated with anti-SLAM (Fig. 13C), but in the absence of *c-fyn* SLAM did not bind SHP-2. Introduction of SAP, together with SLAM and *c-fyn*, completely blocked the interaction of SHP-2 and SLAM. The absence of SHP-2 in the presence of SAP could not be due to lower levels of phosphorylation of SLAM, because a significantly larger percentage of SLAM molecules was phosphorylated in the presence of SAP than in its absence. These results were confirmed in reciprocal co-immunoprecipitation studies with anti-SAP reagents. By contrast, as shown in Fig. 13D, mutant SAP 32R⇒Q did not block the binding of SHP-2 to SLAM. A similar result was obtained with the A1 deletion mutant. Thus, recruitment of SHP-2 to phosphorylated SLAM was blocked by the binding of SAP to the sequence surrounding the most membrane proximal tyrosine residue Y281 of SLAM.

Since SHP-2 itself can act as a negative regulator of signal transduction cascades (Marengere *et al.*, *supra*), binding of SAP could have a positive effect on co-stimulation by anti-SLAM. To test this, SAP was over-expressed by transient transfections into the Jurkat T cells, together with an IL-2-promoter-luciferase reporter construct and SLAM. cDNAs coding for SLAM and SAP were transiently transfected with an IL-2 promoter-luciferase constructs into Jurkat-Tag cells, as described (Martinez-Martinez, S. *et al.*, Mol Cell Biol. 17: 6437-6447, 1997). After 24 hours, transfected cells were stimulated with anti-CD3 and/or anti-SLAM monoclonal antibodies, as described (Cocks *et al.*, *supra*). Postnuclear lysates were analyzed in a Berthold luminometer 8 hours later.

Stimulation of SLAM+SAP transfected cells with a combination of anti-SLAM and anti-CD3 monoclonal antibodies resulted in a significantly higher IL-2-promoter-luciferase reporter activation, than that observed in Jurkat cells transfected

with SLAM alone (with an IL-2-promoter-luciferase reporter construct) (Fig. 14). Anti-SLAM by itself did not induce the IL-2-promoter-luciferase reporter signal, and the signal induced by anti-CD3 reagents was lower than that generated by the two antibodies together. Thus, over-expression of SAP in T cells had a positive effect on the co-stimulatory activity of anti-SLAM.

EXAMPLE VI

Mapping the SAP gene

Given our finding that the SAP gene mapped to the human XLP locus, we next produced a finer map of both the human and the murine SAP genes. This finer mapping allows the identification of putative transcription factors that may bind to the SAP gene and, thus, control SAP protein expression.

On Figs. 15-18 are shown the DNA sequences of the 5' region / exon1 / intron1 of the murine SAP gene (SEQ ID NO: 14), the DNA sequences of exon2 / intron2 of the murine SAP gene (SEQ ID NO: 15), and the DNA sequences of intron2 / exon3 / intron3 / exon4 / intron4 of the murine SAP gene (SEQ ID NO: 16), respectively. From these sequences, we constructed a schematic diagram showing the locations of the four exons of the murine SAP gene on the X chromosome. This information allows the production of a SAP knock-out animal, or a transgenic animal expressing a mutant or truncated SAP protein (see above). Methods for generating such animals are well known (see for example, Fung-Leung *et al.*, Cell 65:443-9, 1991; Kozieradzki *et al.*, J. Immunol. 158:3130-3139, 1997; Galli-Taliadoros *et al.*, J. Immunol. Methods 181:1-15, 1995).

In addition, as shown on Fig. 19, we identified a number of putative binding sites for transcription factors in the non-coding regions of both human and murine SAP (*i.e.*, the 5' untranslated regions and the introns) which may regulate the expression of SAP. By specifically disrupting one or more of these binding sites, the level of expression of SAP may be altered in a patient suffering from a disease

characterized by an inappropriate amount of SH2-domain containing protein mediated signal transduction.

EXAMPLE VII

Higher Expression of SAP in Th1 than Th2 helper T cells

Because engagement of SLAM during antigen-specific T-cell stimulation has been shown to induce IFN-γ production and to redirect the Th2 phenotype to a Th1/Th0 phenotype (Carballido *et al.*, J. Immunol. 159: 4316-4321, 1997), we next sought to determine if Th1 and Th2 helper T cells had different levels of expression of SAP. Accordingly, we isolated Th1 and Th2 cells from mice according to standard techniques. Briefly, $CD4^+$ T cells from mice expressing the transgene for the DO11.10 αβ-TCR, which recognizes residues 323-339 of chicken ovalbumin (OVA) in association with $I\text{-}A^d$, were cultured in complete RPMI 1640 media with OVA 323-339 peptide (1 μM) and mitomycin-treated splenocytes. For Th1 phenotype T cell development, recombinant murine IL-12 (10 ng/ml), neutralizing anti-IL-4 monoclonal antibody (clone 11B11, 40 μg/ml, commercially available from R&D Ssytems, Minneapolis, MN) was added. For Th2 phenotype T cell development, recombinant murine IL-4 (10 ng/ml) and neutralizing polyclonal anti-murine IL-12 (clone TOSH-2, 3 μg/ml, commercially available from Endogen, Cambridge, MA) was added.

Cells were cultured for three rounds of antigenic stimulations under polarizing conditions, and then stimulated on plate-bound anti-CD3 (clone 2C11, commercially available from PharMingen, San Diego, CA) in the presence of human IL-2 (commercially available from Endogen) for 6 hours. After no stimulus or stimulus for 6 hours with, polyA+ RNA was prepared from the cells (using the FastTrack mRNA kit commercially available from Invitrogen, Carlsbad, CA), electrophoretically resolved, and subjected to Northern blotting analyses using as probes the following radiolabelled murine DNAs: Interferon-gamma (IFN-γ; to identify Th1 cells), interleukin-4 (IL-4; to identify Th2 cells), mSAP (which identifies a 0.9 kB band),

mSLAM (which identifies a 2.1 kB band) and β-actin (control; identifies a 2.2 kB band).

As shown on Fig. 20, following 6 hours of stimulation, the 2.1 kB SLAM mRNA band increased in both stimulated (vs. non-stimulated) Th1 and Th2 cells. The 0.9 kB SAP mRNA, however decreased in both Th1 and Th2 cells following 6 hours of stimulation (note that the decrease was less noticeable in the Th1 cell population). However, it is clear that in non-stimulated cells, the level of SAP mRNA species was higher in Th1 cells than in Th2 cells. Given this finding, in order to shift the immune response to a Th1-mediated response as opposed to a Th2-mediated response (*e.g.*, in the treatment of leprosy), it is advisable to treat the patient with a compound that causes an increase in the level of expression of the SAP mRNA in resting T cells.

EXAMPLE VIII

Identification of EAT-2 as a SAP family member protein

The murine EAT-2 protein was identified as an SH2 aberrantly expressed in Ewing's sarcoma tumor cell lines (Thompson *et al.*, Oncogene 13:2649-58, 1996; GenBank Accession Nos. AF020264 (human, fragment) and AF020263 (mouse)). Given our finding of the high sequence identity between the SH2 domain of EAT-2 with SAP, as well as the similarity in the overall length of the proteins, we sought to determine if EAT-2 was a second member of the SAP family of proteins.

To do this, we subcloned the entire coding region of the EAT-2 cDNA into a FLAG-fusion expression vector cloned in expression vector pCMV2-FLAG (commercially available from KODAK) to create a EAT2-FLAG fusion protein terminally fused to the FLAG tag. As in Example IV, the EAT2-FLAG encoding expression vector was co-transfected with the CD8SLAM3 encoding vector into COS cells. Following cell surface biotinylation using the EZ-Link Sulfo-NHS-Biotin reagent (Pierce Chemical Co.), the COS cells were lysed and immunoprecipitated with anti-CD8 antibody. The immunoprecipitates were resolved by SDS-Page and

subjected to Western blotting analyses with anti-FLAG antibody, anti-SAP antibody, or streptavidin. As can be seen on Fig. 21, both Flag tagged SAP and Flag tagged EAT2 can be pulled down with CD8SLAM3 (Fig. 21, top panel). Thus, EAT2, like SAP, binds the cytoplasmic tail of SLAM3, which bears only the one tyrosine residues, Y281. Equivalent loading of the lanes is shown at the bottom panel of Fig. 21, with equivalent binding of streptavidin to all three lanes.

EXAMPLE IX

Isolation of SAP associated proteins by yeast 2-hybrid analysis

A plasmid which encodes the GAL4 DNA-binding domain fused to the entire coding domain of human or murine SAP, is constructed by inserting DNA encoding the coding region of human or SAP into a GAL4 DNA binding domain fusion protein expression vector pGBT9 (Clontech, Palo Alto, CA). GAL4 DNA binding domain-SAP fusion protein is then used as "bait" (DNA-binding domain hybrid) in yeast two-hybrid screens of a human cDNA "prey" library of PHA-stimulated peripheral blood leukocytes (PBL) or a murine cDNA "prey" library of a T cell lymphoma (both commercially available from Clontech). The yeast two-hybrid assay and isolation of positive clones and subsequent interaction analyses are carried out as has been previously described (see, for example, PCT Publication WO 95/28497).

DNA sequencing of positive SAP-interacting clones is performed on an Applied Biosytems model 373A automated DNA sequencer. Full length cDNA encoding polypeptide fragments of SAP-interacting proteins are isolated by using the cDNA clone encoding the SAP-interacting polypeptide fragment to screen other libraries (*e.g.*, longer cDNA libraries or genomic libraries) using standard hybridization techniques (see Ausubel *et al.*, *supra*; Sambrook *et al.*, *supra*).

EXAMPLE X

Additional SH2 Domain-Containing Protein-Mediated Signal Transduction Assays

Numerous assays are known which evaluate SH2 domain-containing protein

mediated signal transduction. Such signal transduction pathways occur in, for example, epidermal cells through the Epidermal Growth Factor (EGF) receptor (Vogel *et al.*, Science 259: 1611-1614, 1993), fibroblasts through the Platelet Derived Growth Factor (PDGF) receptor (Fantl *et al.*, Cell 69: 413-423, 1992), and B cells (Ono *et al.*, Cell 90: 293-301, 1997).

In one particular example, SAP activity on antigen-specific T cell activation can be assessed using a variety of antigen-specific T cell activation assays are known in the art. Most are based upon the increased production of activation dependent T cell genes including, without limitation, the following cell surface molecules: Fas ligand, MHC class II, CD25, and CD69; as well as the following cytokines: interleukin-2 (IL-2), and γ-interferon. All of the following examples may be easily modified to identified compounds which affect SAP expression and biological activities, as well as peptide and non-peptide SAP mimetics which have SAP biological activity.

For example, a T cell specific for, for example, a viral peptide, may be stimulated with a syngeneic antigen presenting cell presenting that viral peptide on its cell surfaces in context with MHC. An activated T cell will express Fas ligand, MHC class II, CD25, and CD69 on its cell surface (as opposed to a resting, unactivated T cell which expresses none of these cell surface molecules). Similarly, an activated T cell, unlike a resting T cell, will produce IL-2 and γ-IFN. The cell surface activation markers can be detected by antibodies (commercially available from, for example, Becton Dickinson, San Jose, CA). Binding of these antibodies may be rapidly assessed by binding of a secondary labeled antibody, followed by detection of that bound secondary antibody (by, for example, FACS analysis or addition of colorimetric substrate for the label). The cytokines released by activated T cells may be detected from the supernatants of cells by commercially available ELISA kits (commercially available from, for example, Endogen Inc., Woburn, MA).

Antigen-specific T cell activation may also be determined by the production of activation-dependent cytokines, such as IL-2, in a proliferation assay of IL-2

dependent cells. For example, Jurkat human T cells may be stimulated with anti-CD3 plus anti-CD28 antibodies (commercially available from, for example, Pharmingen, San Diego, CA). Following culture for 24-48 hours at 37°C, the supernatants from these cells is collected, freeze-thawed, and added to an IL-2 dependent cell line, such as CTLL-20 (available from the ATCC). For proliferation studies, the CTLL-20 cells are then returned to culture for 48-72 hours total, with the addition of ^{3}H-thymidine to the cells in the final 6-20 hours of culture. The cells are then lysed, and proliferation was measured by amount of 3H-thymidine uptake. Non-radioactive cell proliferation assays are also known in the art (*e.g.*, the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay commercially available from Promega Co., Madison, WI).

Other examples of antigen-specific T cell activation assays are also provided in the following references: increased interleukin-2 (IL-2) production (Aversa *et al.*, J. Immunol. 1588: 4036-4044, 1997); increased interferon-γ (IFN-γ) production (Aversa *et al.*, J. Immunol. 1588: 4036-4044, 1997); increased CD69 expression (Zubiaur *et al.*, J. Immunology 159: 193-205, 1997); increased Fas Ligand expression (Ju *et al.*, Nature 373:444-448, 1995); increased interleukin-4 production (Cocks *et al.*, *supra*); increased incidence of phosphotyrosine-containing proteins (Zubiaur *et al.*, *supra*); and recruitment of phosphatidylinositol 3'-kinase (PI3-kinase) to the T cell receptor signaling complex (de Aos *et al.*, J. Biol. Chem. 272: 25310-25318, 1997).

Other Embodiments

In other embodiments, the invention includes any protein which is substantially identical to the human SAP polypeptide provided in Fig. 2B (SEQ ID NO: 4), murine SAP polypeptide provided in Fig. 2D (SEQ ID NO: 6), or to GenBank Accession Nos. AF020263 and AF020264; such homologues include other substantially pure naturally-occurring mammalian SAP family member proteins as well as splice variants, allelic variants; natural mutants; induced mutants; DNA sequences which encode proteins and also hybridize to the SAP family member encoding DNA sequence of Fig. 2A (SEQ ID NO: 3), Fig. 2C (SEQ ID NO: 5),

GenBank Accession No. AF020263, or GenBank Accession No. AF020264, under high stringency conditions (*e.g.*, hybridizing at 2X SSC at 40°C with a probe length of at least 40 nucleotides) or, less preferably, under low stringency conditions (*e.g.*, hybridizing at 5X SSC at 25°C with a probe length of at least 80 nucleotides); and proteins specifically bound by antisera directed to a SAP family member polypeptide. The term also includes chimeric polypeptides that include a portion derived from a SAP family member polypeptide.

The invention further includes analogs of any naturally-occurring SAP family member polypeptides. Analogs can differ from the naturally-occurring SAP family member proteins by amino acid sequence differences, by post-translational modifications, or by both. Analogs of the invention will generally exhibit at least 50%, more preferably 70%, and most preferably 90% or even 95% identity with all or part of a naturally-occurring SAP family member amino acid sequence. The length of sequence comparison is at least 15 amino acid residues, preferably at least 25 amino acid residues, and more preferably more than 35 amino acid residues. Modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, *e.g.*, acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring SAP family member polypeptide by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook *et al.*, *supra*; or Ausubel *et al.*, *supra*). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, *e.g.*, D-amino acids or non-naturally-occurring or synthetic amino acids, *e.g.*, B or Y amino acids. In addition to full-length polypeptides, the invention also includes SAP family member polypeptide fragments. As used herein, the term "fragment," means at least 20 contiguous amino acids, preferably at least 30 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least

60 to 80 or more contiguous amino acids. Polypeptide fragments of SAP family members can be generated by methods known to those skilled in the art or may result from normal protein processing (*e.g.*, removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

Preferable fragments or analogs according to the invention are those which facilitate specific detection of a SAP family member nucleic acid or amino acid sequence in a sample to be diagnosed. Particularly useful SAP family member polypeptide fragments for this purpose include, without limitation, the amino acid fragments corresponding to the SH2 domain.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

Other embodiments are within the claims.

What is claimed is:

Claims

1. A substantially pure nucleic acid encoding a SAP polypeptide.

2. The nucleic acid of claim 1, wherein said SAP polypeptide is a fragment of the full length naturally-occurring SAP polypeptide.

3. The nucleic acid of claim 1, wherein said nucleic acid is operably linked to a second nucleic acid.

4. The nucleic acid of claim 3, wherein said second nucleic acid is a coding sequence and said nucleic acid operably linked to said second nucleic acid produces a fusion protein comprising said SAP polypeptide.

5. The nucleic acid of claim 3, wherein said second nucleic acid is a gene promoter.

6. The nucleic acid of claim 1, wherein said nucleic acid comprises a mutation, said mutation resulting in an amino acid alteration in said SAP polypeptide.

7. The nucleic acid of claim 1, wherein said nucleic acid comprises a nucleic acid sequence that is substantially identical to SEQ ID NO: 3 or is substantially identical to SEQ ID NO: 5.

8. The nucleic acid of claim 1, wherein said nucleic acid comprises a nucleic acid sequence encoding a naturally-occurring SH2 domain.

9. The nucleic acid of claim 1, wherein said polypeptide has SAP biological activity.

10. The nucleic acid of claim 1, wherein said polypeptide modulates SH2 domain-containing protein-mediated signal transduction.

11. A substantially pure SAP polypeptide.

12. The polypeptide of claim 11, wherein said polypeptide is a fragment of the full length naturally-occurring SAP polypeptide.

13. The polypeptide of claim 11, wherein said polypeptide is part of a fusion protein.

14. The polypeptide of claim 11, wherein said polypeptide comprises an amino acid sequence that has a mutation as compared to the naturally-occurring amino acid sequence of said polypeptide.

15. The polypeptide of claim 11, wherein said polypeptide comprises an amino acid sequence that is substantially identical to SEQ ID NO: 4 or is substantially identical to SEQ ID NO: 6.

16. The polypeptide of claim 11, wherein said polypeptide comprises a naturally-occurring SH2 domain.

17. The polypeptide of claim 11, wherein said polypeptide has SAP biological activity.

18. The polypeptide of claim 11, wherein said polypeptide modulates SH2 domain-containing protein-mediated signal transduction.

19. A method of treating a disease involving aberrant SH2 domain-containing

protein-mediated signal transduction in a patient, said method comprising administering to said patient a SAP family member polypeptide or a fragment, mutant, or fusion thereof.

20. The method of claim 19, wherein said polypeptide comprises an amino acid sequence substantially identical to SEQ ID NO: 4 or is substantially identical to SEQ ID NO: 6.

21. The method of claim 19, wherein said polypeptide is an EAT-2 polypeptide.

22. A method of identifying a compound that modulates SH2 domain-containing protein-mediated signal transduction, said method comprising:

(a) providing a cell comprising a SAP family member polypeptide-encoding gene;

(b) contacting said cell with a candidate compound; and

(c) monitoring expression of said SAP family member polypeptide-encoding gene, an alteration in the level of said expression of said gene in response to said candidate compound indicating the presence of a compound that modulates SH2 domain-containing protein-mediated signal transduction.

23. A method of identifying a compound that modulates SH2 domain-containing protein-mediated signal transduction, said method comprising:

(a) providing a cell comprising a reporter gene operably linked to a promoter from a SAP family member polypeptide-encoding gene;

(b) contacting said cell with a candidate compound; and

(c) measuring expression of said reporter gene, an alteration in the level of said expression of said reporter gene in response to said candidate compound indicating the presence of a compound that modulates SH2 domain-containing

protein-mediated signal transduction.

24. The method of claim 22 or 23, wherein said SAP family member polypeptide-encoding gene encodes a SAP polypeptide or encodes an EAT-2 polypeptide.

25. A method of identifying a compound that modulates SH2 domain-containing protein-mediated signal transduction, said method comprising:

(a) providing a cell having:

(i) a reporter gene operably linked to a DNA-binding-protein recognition site;

(ii) a first fusion gene capable of expressing a first fusion protein, said first fusion protein comprising a SAP family member polypeptide covalently bonded to a binding moiety, said binding moiety capable of specifically binding to said DNA-binding-protein recognition site; and

(iii) a second fusion gene capable of expressing a second fusion protein, said second fusion protein comprising a SLAM polypeptide covalently bonded to a gene activating moiety;

(b) exposing said cell to a candidate compound; and

(c) measuring reporter gene expression in said cell, an alteration in the level of said expression of said reporter gene in response to said candidate compound indicating the presence of a compound that modulates SH2 domain-containing protein-mediated signal transduction.

26. A method of identifying a compound that modulates SH2 domain-containing protein-mediated signal transduction, said method comprising:

(a) providing a cell having:

(i) a reporter gene operably linked to a DNA-binding-protein recognition site;

(ii) a first fusion gene capable of expressing a first fusion protein, said first fusion protein comprising a SLAM polypeptide covalently bonded to a binding moiety, said binding moiety capable of specifically binding to said DNA-binding-protein recognition site; and

(iii) a second fusion gene capable of expressing a second fusion protein, said second fusion protein comprising a SAP family member polypeptide covalently bonded to a gene activating moiety;

(b) exposing said cell to said compound; and

(c) measuring reporter gene expression in said cell, an alteration in the level of said expression of said reporter gene in response to said candidate compound indicating the presence of a compound that modulates SH2 domain-containing protein-mediated signal transduction.

27. A method of identifying a compound as an SAP family member protein mimetic, said method comprising the steps of:

(a) providing a SLAM polypeptide, or a fragment or fusion thereof, said SLAM polypeptide bearing non-phosphorylated tyrosine residues;

(b) contacting said SLAM polypeptide with a SAP family member polypeptide;

(c) contacting said SLAM polypeptide and said SAP family member polypeptide with a candidate compound; and

(d) measuring level of interaction of said SLAM polypeptide with said SAP family member polypeptide, a decrease in said level in response to said compound relative to a level not contacted with said compound indicating said compound is a SAP family member protein mimetic.

28. The method of claim 25, 26, or 27, wherein said SAP family member polypeptide is a SAP protein or an EAT-2 protein.

29. A method of identifying a compound as an SAP family member protein mimetic, said method comprising the steps of:

(a) providing a SLAM polypeptide, or a fragment or fusion thereof, said SLAM polypeptide bearing non-phosphorylated tyrosine residues; and

(b) contacting said SLAM polypeptide with a candidate compound, wherein said candidate compound binding to said SLAM polypeptide indicates said candidate compound is a SAP family member protein mimetic.

30. The method of claim 27 or 29, wherein said SLAM polypeptide is bound to a solid state substrate.

31. A method of identifying a polypeptide that modulates SH2 domain-containing protein-mediated signal transduction, said method comprising:

(a) providing a cell having:

(i) a reporter gene operably linked to a DNA-binding-protein recognition site;

(ii) a first fusion gene capable of expressing a first fusion protein, said first fusion protein comprising a SAP family member polypeptide covalently bonded to a binding moiety, said binding moiety capable of specifically binding to said DNA-binding-protein recognition site; and

(iii) a second fusion gene capable of expressing a second fusion protein, said second fusion protein selected from a library, said library comprising a polypeptide covalently bonded to a gene activating moiety, said polypeptide encoded by a cDNA of said library; and

(b) measuring reporter gene expression in said cell, an increase in said reporter gene expression identifying the presence of a polypeptide that modulates SH2 domain-containing protein-mediated signal transduction.

32. A method of modulating SH2 domain-containing protein-mediated signal

transduction in a mammal, said method comprising providing a transgene encoding a SAP family member polypeptide or fragment thereof to a cell of said mammal, said transgene being positioned for expression in said cell.

33. A method of modulating SH2 domain-containing protein-mediated signal transduction in a mammal, said method comprising administering to a cell of said mammal a compound which modulates the biological activity of a SAP family member polypeptide.

34. A method of increasing antigen-specific T cell activation in a mammal, said method comprising providing a transgene encoding a SAP family member polypeptide, said transgene being positioned for expression in said cell.

35. A method of increasing antigen-specific T cell activation in a mammal, said method comprising administering to a cell of said mammal a compound which increases the biological activity of a SAP family member polypeptide.

36. The method of claim 34 or 35, wherein said antigen-specific T cell activation is mediated by Th1 helper T cells.

37. The method of claim 31, 32, 33, 34, or 35, wherein said SAP family member polypeptide is a SAP protein or an EAT-2 protein.

38. A transgenic mammal, said mammal having a knockout mutation in an endogenous SAP family member protein-encoding nucleic acid sequence.

39. A transgenic mammal, said mammal having an exogenous SAP family member protein-encoding nucleic acid sequence operably linked to a promoter.

40. The transgenic mammal of claim 38 or 39, wherein said SAP family member protein is a SAP protein or an EAT-2 protein.

41. The transgenic mammal of claim 38 or 39, wherein said animal has aberrant SH2 domain-containing protein mediated signal transduction.

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1   /   1                                    31   /   11
cag ttg aga aga aga ggt aaa acg aac cat tac cag aca aca gtg gaa aaa aaa agc ctt
gln leu arg arg arg gly lys thr asn his tyr gln thr thr val glu lys lys ser leu
61   /   21                                  91   /   31
acg atc tat gcc caa gtc cag aaa cca ggt cct ctt cag aag aaa ctt gac tcc ttc cca
thr ile tyr ala gln val gln lys pro gly pro leu gln lys lys leu asp ser phe pro
121  /   41                                  151  /   51
gct cag gac cct tgc acc acc ata tat gtt gct gcc aca gag cct gtc cca gag tct gtc
ala gln asp pro cys thr thr ile tyr val ala ala thr glu pro val pro glu ser val
181  /   61                                  211  /   71
cag gaa aca aat tcc atc aca gtc tat gct agt gtg aca ctt cca gag agc
gln glu thr asn ser ile thr val tyr ala ser val thr leu pro glu ser
```

Figure 1

HUMAN SAP

DNA

GGTTGACTTGTGCCTGGCTGCAGTAGCAGCGGCATCTCCCTTGCACAGTTCTCCTCCTCGG
CCTGCCCAAGAGTCCACCAGGCCATGGACGCAGTGGCTGTGTATCATGGCAAAATC
AGCAGGGAAACCGGCGAGAAGCTCCTGCTTGCCACTGGGCTGGATGGCAGCTAT
TTGCTGAGGGACAGCGAGAGCGTGCCAGGCGTGTACTGCCTATGTGTGCTGTAT
CACGGTTACATTTATACATACCGAGTGTCCCAGACAGAAACAGGTTCTTGGAGT
GCTGAGACAGCACCTGGGGTACATAAAAGATATTTCCGGAAAATAAAAAATCTC
ATTTCAGCATTTCAGAAGCCAGATCAAGGCATTGTAATACCTCTGCAGTATCCA
GTTGAGAAGAAGTCCTCAGCTAGAAGTACACAAGGTACTACAGGGATAAGAGAA
GATCCTGATGTCTGCCTGAAAGCCCCATGAAGAAAAATAAAACACCTTGTACTTTAT
TTTCTATAATTTAAATATATGCTAAGTCTTATATATTGTAGATAATACAGTTCGGTGAGCT
ACAAATGCATTTCTAAAGCCATTGTAGTCCTGTAATGGAAGCATCTAGCATGTCGTCAAAG
CTGAAATGGACTTTTGTACATAGTGAGGAGCTTTGAAACGAGGATTGGGAAAAAGTAATTC
CGTAGGTTATTTTCAGTTATTATATTTACAAATGGGAAACAAAAGGTAATGAATACTTTAT
AAAGGATTAATGTCAATTCTTGCCAAATATAAATAAAAATAATCCTCAGTTTTTGTGAAAA
GCTCCATTTTTAGTGAAATATTATTTTATAGCTACTAATTTTAAAATGTCTTGCTTGATTG
TATGGTGGGAAGTTGGCTGGTGTCCCTTGTCTTTGCCAAGTTCTCCACTAGCTATGGTGTC
ATAGGCTCTTTTGGGATTTTTGAAGCTGTATACTGTGTGCTAAAACAAGCACTAAACAAAG
AGTGAAGGATTTATGTTTAATTCTGAAAGCAACCTTCTTGCCTAGTGTTCTGATATTGGAC
AGTAAAATCCACAGACCAACCTGGAGTTGAAAATCTTATAATTTAAAATATGCTCTAAACA
TGTTTATCGTATTTGATGCTACAGGATTTGAAATTGTATTACAAATCCAATGAAATGAGTT
TTTCTTTTCATTTACCTCTGCCCCAGTTGTTTCTACTACATGGAAGACCTCATTTTGAAGG
GAAATTTCAGCAGCTGCAGCTCATGAGTAACTGATTTGTAACAAGCCTCCTTTTAAAGTAA
CCCTACAAAACCACTGGAAAGTTTATGGTTGTATTATTTTTAAAAAAAATTCCAAGTGATT
GAAACCTACACGAGATACAGAATTTTATGCGGCATTTTCTTCTCACATTTATATTTTTGTG
ATTTTGTGATTGATTATATGTCACTTTGCTACAGGGCTCACAGAATTCATTCACTCAACAA
ACATAATAGGGCGCTGAGGGCATAGAAGTAAAAACACCTGGTCCCTGCTCTCAGTTCACTG
TCTTGTTGGACGAGAAAACAATAACGATAAAAGACAGTGAAAGAAAATAACGATAAAAGAC
AGTGAAAGAAAATAACAATAAAAGACAAGGAAAAAATAACAATGAAAGTTGATAAGTACAT
GATAAGCGAGGTTCCCCGTGTGTAGGTAGATCTGGTCTTTAGAGGCAGATAGATAGGTCAG
TGCAAATACTCTGGTCCATGGGCCATATGAAAAGGCTAAGCTTCACTGTAAAATAATAACT
GGGAATTCTGGATTGTGTATGGGTGTTGGTGAACTTGGTTTTAATTAGTGAACTGCTGAGA
GACAGAGCTATTCTCCATGTACTGGCAAGACCTGATTTCTGAGCATTTAATATGGATGCCG
TGGGAGTACAAAAGTGGAGTGTGGCCTGAGTAATGCATTATGGGTGGTTTACCATTTCTTG
AGGTAAAAGCATCACATGAACTTGTAAAGGAATTTAAAAATCCTACTTTCATAATAAGTTG
CATAGGTTTAATAATTTTTAATTATATGGCTTGAGTTTAAATTGTAATAGGCGTAACTAAT
TTTAACTCTATAATGTGTTCATTCTGGAATAATCCTAAACATATGAATTATGTTTGCATGT
TCACTTCCAAGAGCCTTTTTTTGAAAAAAAGCTTTTTTTGAATCATCAAGTCTTTCACATT
TAAATAAAGTGTTTGAAAGC

Figure 2A

PROTEIN

MDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVLYHGYIYTYRVSQTET
GSWSAETAPGVHKRYFRKIKNLISAFQKPDQGIVIPLQYPVEKKSSARSTQGTTGIREDPD
VCLKAP

Figure 2B

SAP Sequences:

mouse SAP cDNA:
ATGGATGCAGTGACTGTGTACCACGGCAAAATCAGCAGGGAGACCGGGGAGAAGCTCTTACTCGCTACCGGGCTGGAT
GGAAGCTATCTGCTGCGAGACAGCGAGAGTGTCCCTGGCGTGTACTGCCTGTGTGTTTTGTATCAAGGTTACATCTAC
ACATATCGAGTGTCCCAGACAGAAACAGGTTCTTGGAGTGCCGAGACAGCACCTGGAGTACATAAAAGATTTTTCCGG
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TCCTCTGGCAGGGGCCCACAAGCTCCCACAGGGAGAAGAGATTCTGATATCTGCCTGAATGCACCATGA

Figure 2C

mouse SAP protein:
MDAVTVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVLYQGYIYTYRVSQTETGSWSAETAPGVHKRFFR
KVKNLISAFQKPDQGIVTPLQYPVEKSSGRGPQAPTGRRDSDICLNAP

Figure 2D

Mutants

hSAPΔ55 cDNA:
ATGGACGCAGTGGCTGTGTATCATGGCAAAATCAGCAGGGAAACCGGCGAGAAGCTCCTG
CTTGCCACTGGGCTGGATGGCAGCTATTTGCTGAGGGACAGCGAGAGCGTGCCAGGCGTG
TACTGCCTATGTGTGCTGTATCACGGTTACATTTATACATACCGAGTGTCCCAGACAGAA
ACAGGTTCTTGGAGTGCTGAGCATTTCAGAAGCCAGATCAAGGCATTGTAATACCTCTGC
AGTATCCAGTTGAGAAGAAGTCCTCAGCTAGAAGTACACAAGGTACTACAGGGATAAGAG
AAGATCCTGATGTCTGCCTGAAAGCCCCATGA

hSAPΔ55 protein:
MDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVLYHGYIYTYRVSQTE
TGSWSAEHFRSQIKAL

A1-1 cDNA:
ATGGACGCAGTGGCTGTGTATCATGGCAAAATCAGCAGGGAAACCGGCGAGAAGCTCCTG
CTTGCCACTGGGCTGGATGGCAGCTATTTGCTGAGGGACAGCGAGAGCGTGCCAGGCGTG
TACTGCCTATGTGTGCTACAGCACCTGGGGTACATAAAAGATATTTCCGGAAAATAAAAA
ATCTCATTTCAGCATTTCAGAAGCCAGATCAAGGCATTGTAATACCTCTGCAGTATCCAG
TTGAGAAGAAGTCCTCAGCTAGAAGTACACAAGGTACTACAGGGATAAGAGAAGATCCTG
ATGTCTGCCTGAAAGCCCCATGA

A1-1 protein:
MDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVLQHLGYIKDISGK

A1-2 cDNA:
ATGGACGCAGTGGCTGTGTATCATGGCAAAATCAGCAGGGAAACCGGCGAGAAGCTCCTG
CTTGCCACTGGGCTGGATGGCAGCTATTTGCTGAGGGACAGCGAGAGCGTGCCAGGCGTG
TACTGCCTATGTGTGCTCATTTCAGAAGCCAGATCAAGGCATTGTAATACCTCTGCAGTA
TCCAGTTGAGAAGAAGTCCTCAGCTAGAAGTACACAAGGTACTACAGGGATAAGAGAAGA
TCCTGATGTCTGCCTGAAAGCCCCATGA

A1-2 protein:
MDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVLISEARSRHCNTSAVSS

Exon2 genomic mutant:
ggaaactgtggttgggcagatacaatatggagacaagttagattcactatgctttacttc
ctatgaatgcaatgacaccatatacgtgtgtcctagtatatgtgacatttatatgtaata
ttaagctcaaatttaaagtatccattgttcttttggaatctttcagtaatggaagtttat
tctttcaGagGTATCACGGTTACATTTATACATACCGAGTGTCCCAGACAGAAACAGGTT
CTTGGAGTGCTGAGgtatagttgtatttatttttgcttctgggggtgtcaaggaggtatt
tgaaatttaggctggttttataaaagagcaaattatacattattaagtattcataaggtt
taaatctctaaagctccaatccaaaattgttcatggatcattaagaaagctttagagaat
tttggtcccagtcctgtttagcc

Figure 3

The amino acid sequence of human SAP with the consensus motifs of SH2 domains in bold.

```
          10         20         30         40         49
MDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVLYHG
          60         70         80         90         100
YIYTYRVSQTETGSWSAETAPGVHKRYFRKIKNLISAFQKPDQGIVIPLQY
          110        120        128
PVEKKSSARSTQGTTGIREDPDVCLKAP
```

Figure 4A

Concensus motifs found in SH2 domains as defined by a program termed BLOCKS i.e. BLOCK PR00401 SH2 DOMAIN

Internet Address: www.blocks.fhcrc.org
Reference: S Henikoff and JG Henikoff "Protein family classification based on searching a database of blocks *Genomics* 19: 97-107 (1994)

```
BLOCK PR00401A          VYHGKISRETGEKLL          SAPh

BLOCK PR00401B          DGSYLLRDSES              SAPh

BLOCK PR00401C          PGVYCLCVLYHG             SAPh

BLOCK PR00401D          YIYTYRVSQTE              SAPh

BLOCK PR00401E          YFRKIKNLISAFQKP          SAPh
```

Figure 4B

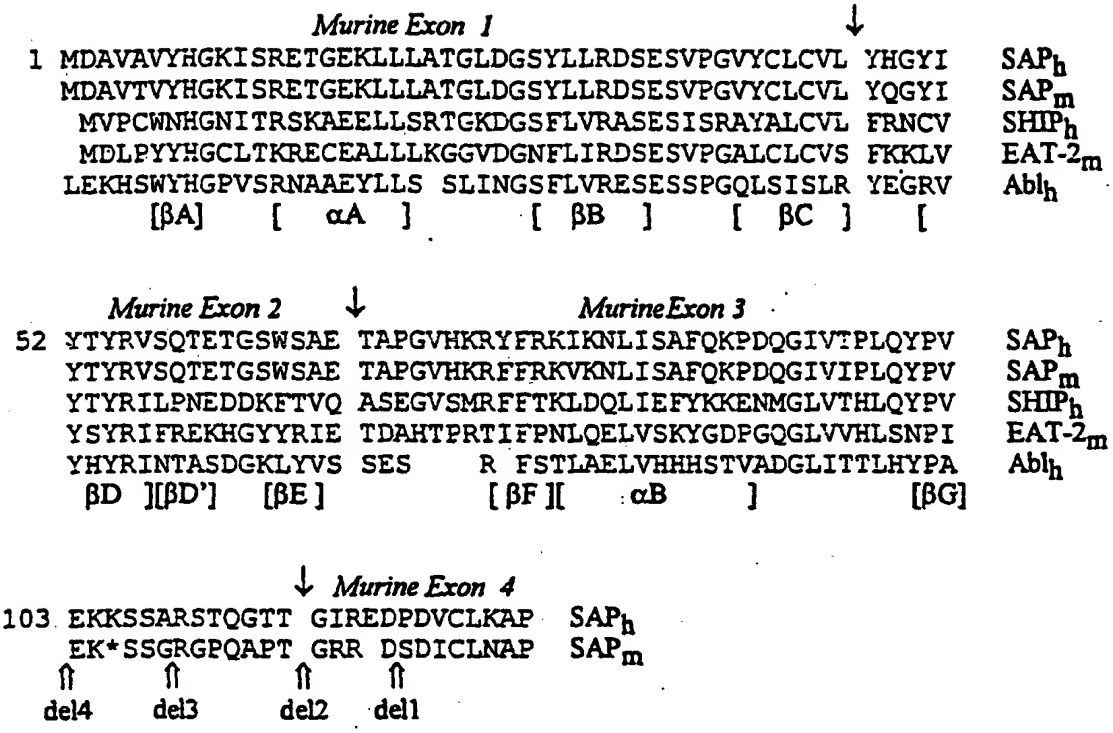

Murine Exon 1
1 MDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVL YHGYI SAPh
MDAVTVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVL YQGYI SAPm
MVPCWNHGNITRSKAEELLSRTGKDGSFLVRASESISRAYALCVL FRNCV SHIPh
MDLPYYHGCLTKRECEALLLKGGVDGNFLIRDSESVPGALCLCVS FKKLV EAT-2m
LEKHSWYHGPVSRNAAEYLLS SLINGSFLVRESESSPGQLSISLR YEGRV Ablh
[βA] [αA] [βB] [βC] [
Murine Exon 2
Murine Exon 3
52 YTYRVSQTETGSWSAE TAPGVHKRYFRKIKNLISAFQKPDQGIVIPLQYPV SAPh
YTYRVSQTETGSWSAE TAPGVHKRFFRKVKNLISAFQKPDQGIVIPLQYPV SAPm
YTYRILPNEDDKFTVQ ASEGVSMRFFTKLDQLIEFYKKENMGLVTHLQYPV SHIPh
YSYRIFREKHGYYRIE TDAHTPRTIFPNLQELVSKYGDPGQGLVVHLSNPI EAT-2m
YHYRINTASDGKLYVS SES R FSTLAELVHHHSTVADGLITTLHYPA Ablh
βD][βD'] [βE] [βF][αB] [βG]
Murine Exon 4
103 EKKSSARSTQGTT GIREDPDVCLKAP SAPh
EK*SSGRGPQAPT GRR DSDICLNAP SAPm
del4
del3
del2
del1

Figure 5

Figure 6A

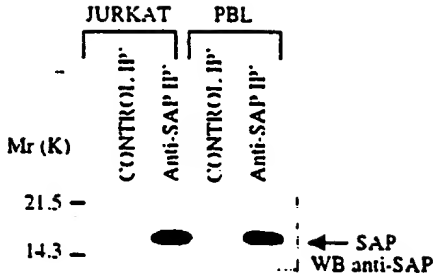


Figure 6B

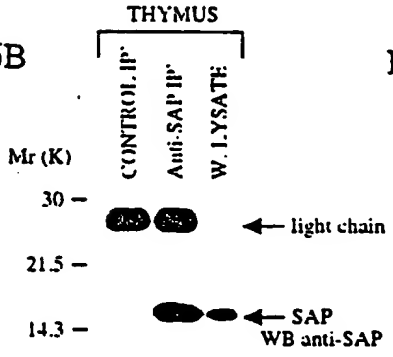


Figure 6C

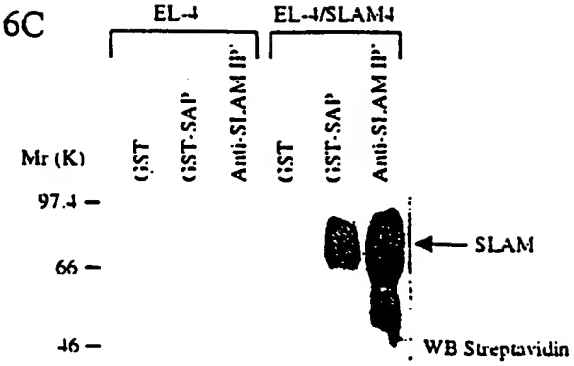


Figure 6D

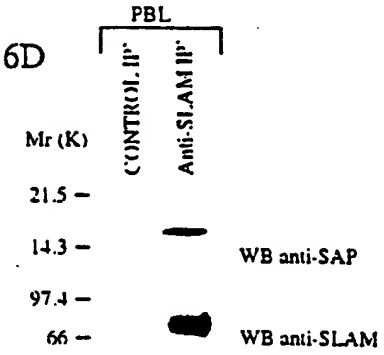


Figure 7A

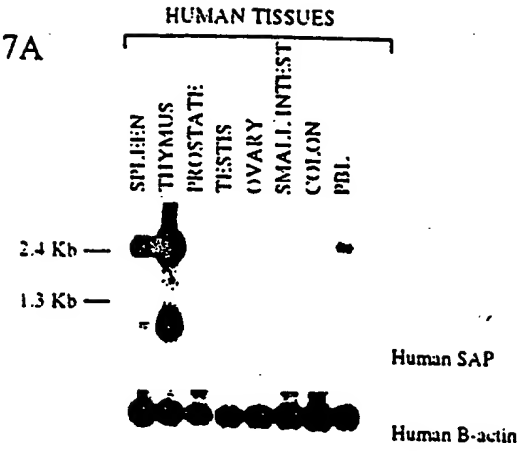


Figure 7B

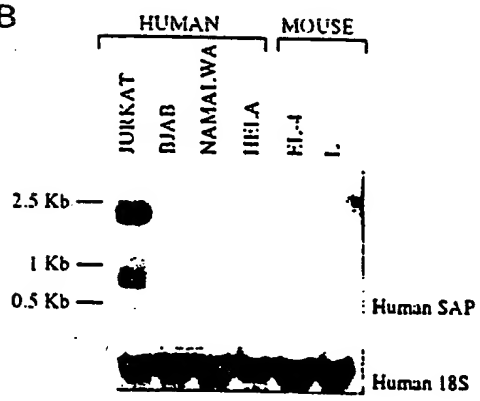


Figure 7C

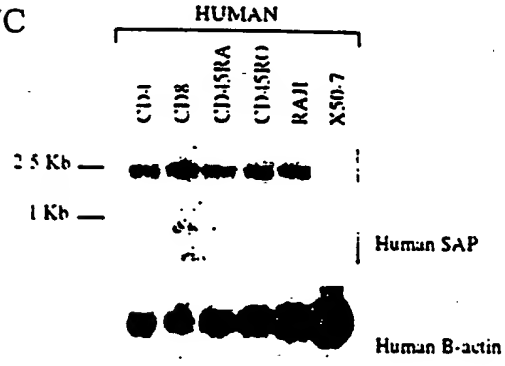


Figure 8A

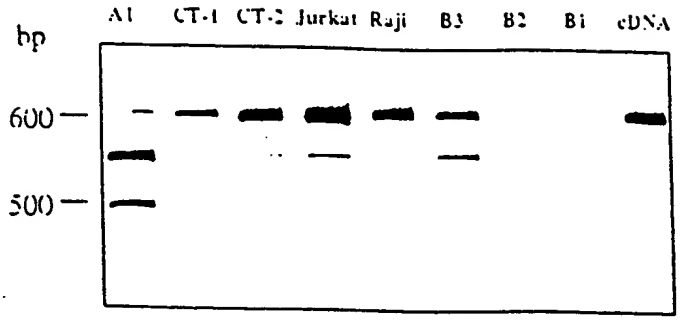


Figure 8B

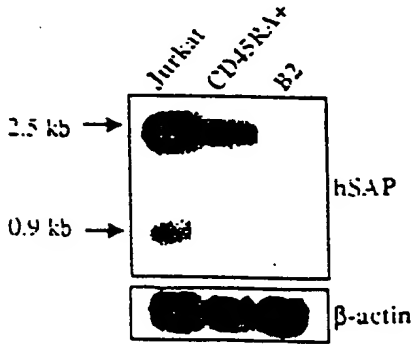


Figure 9A

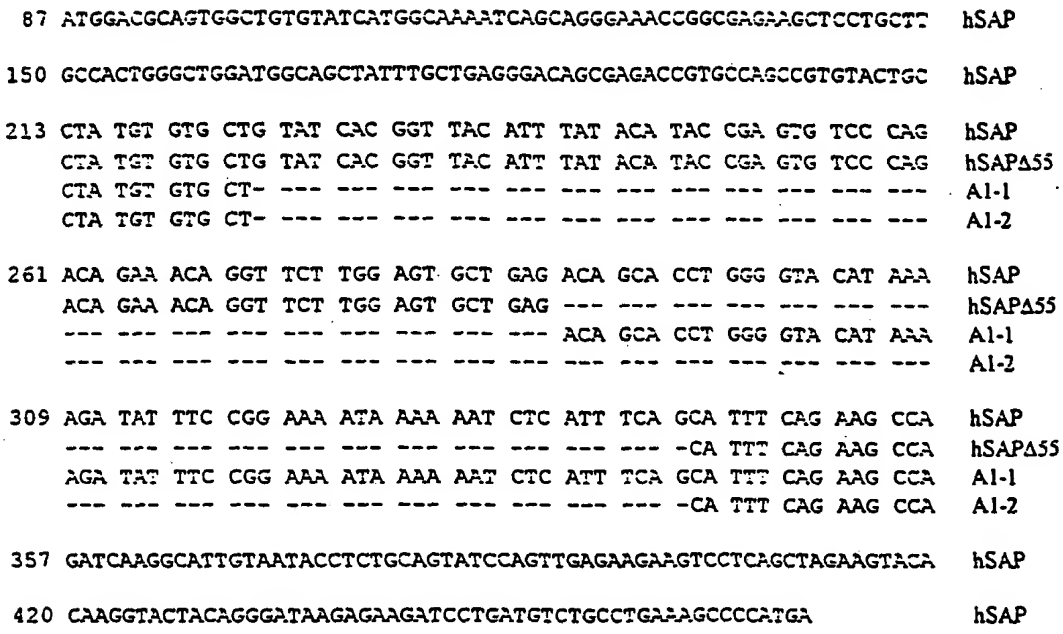


Figure 9B

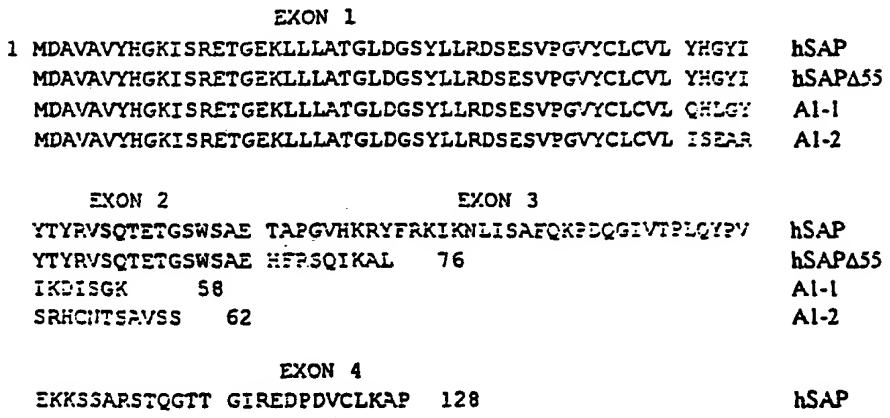


Figure 10A

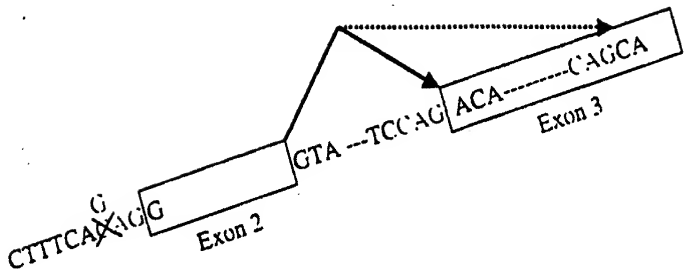
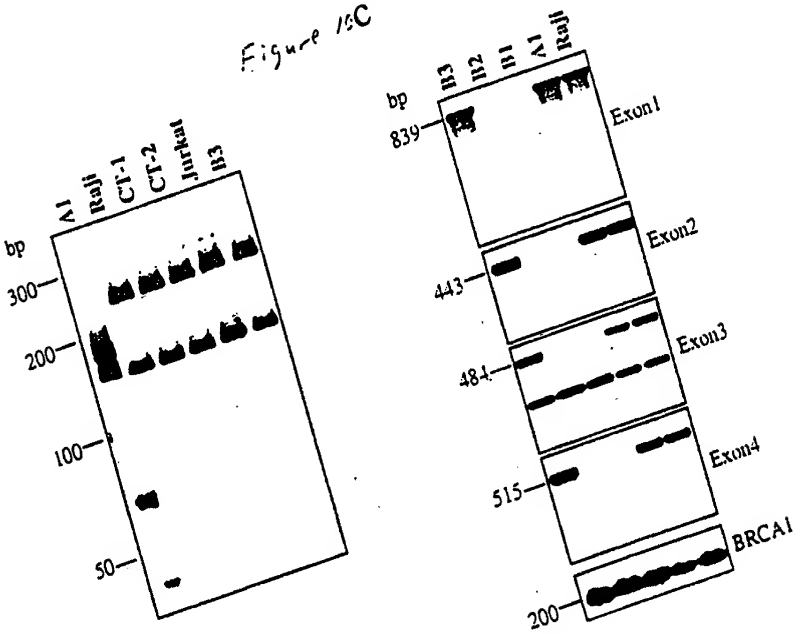


Figure 10C

Figure 10B



CD8SLAM PROTEINS

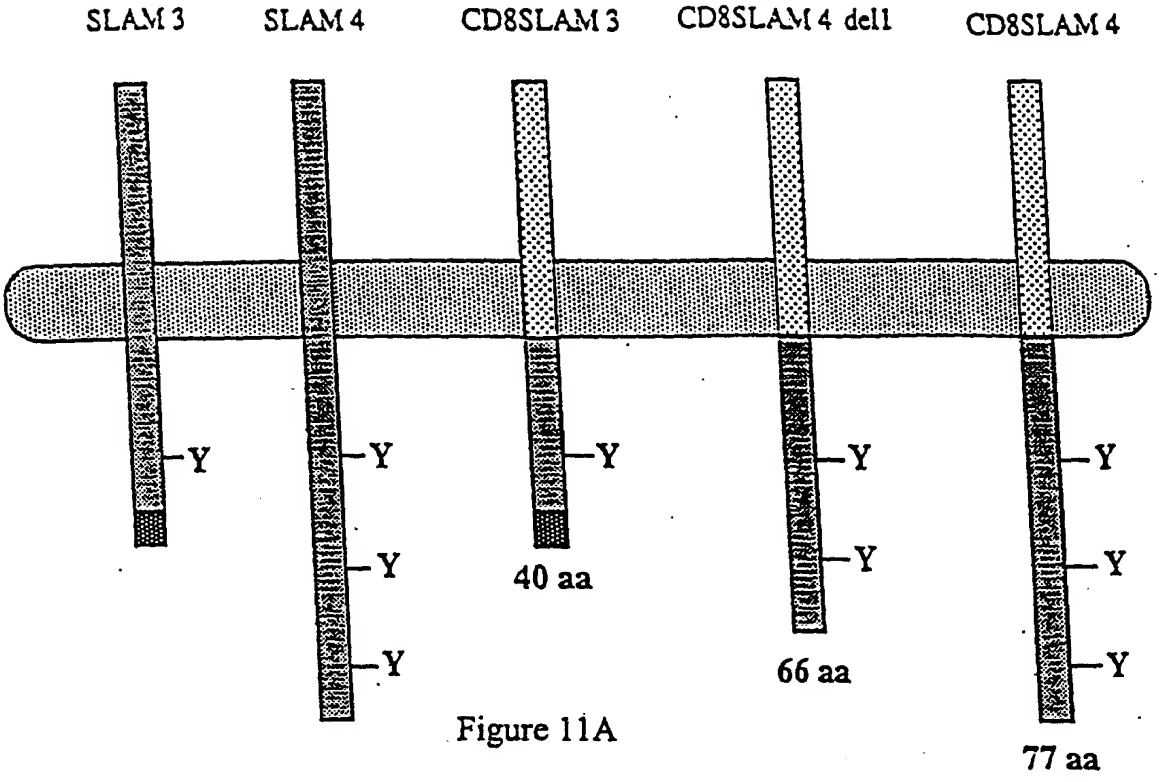


Figure 11A

SLAM 3 QLRRRGKTNHYQTTVEKKSLTIYAQVQKPGDTHHQTSDLF

SLAM 4 del 1 QLRRRGKTNHYQTTVEKKSLTIYAQVQKPGPLQKKLDSFPA
QDPCTTIYVAATEPVPESVQETNSI

SLAM4 QLRRRGKTNHYQTTVEKKSLTIYAQVQKPGPLQKKLDSFPA
QDPCTTIYVAATEPVPESVQETNSITVYASVTLPES

Figure 11B

Figure 12A

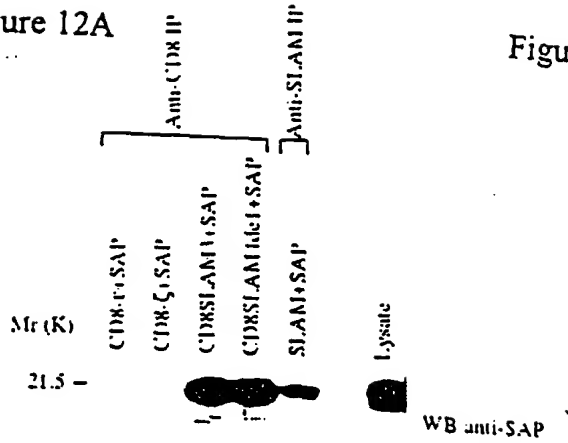


Figure 12B

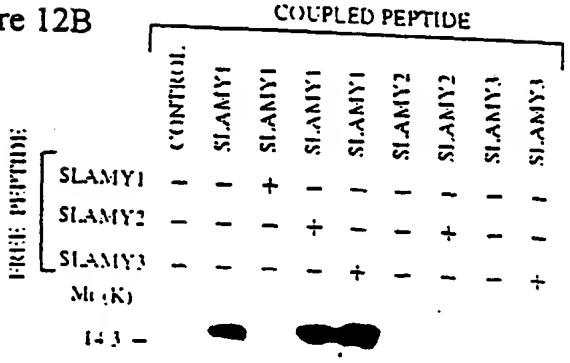


Figure 12C

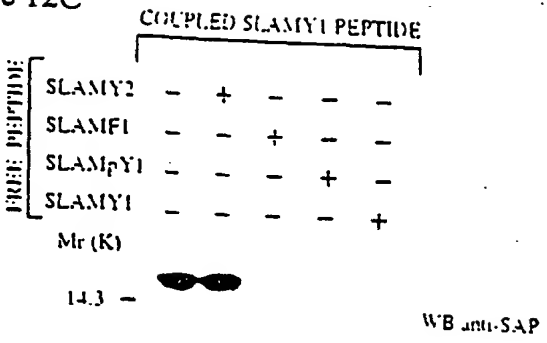


Figure 12D

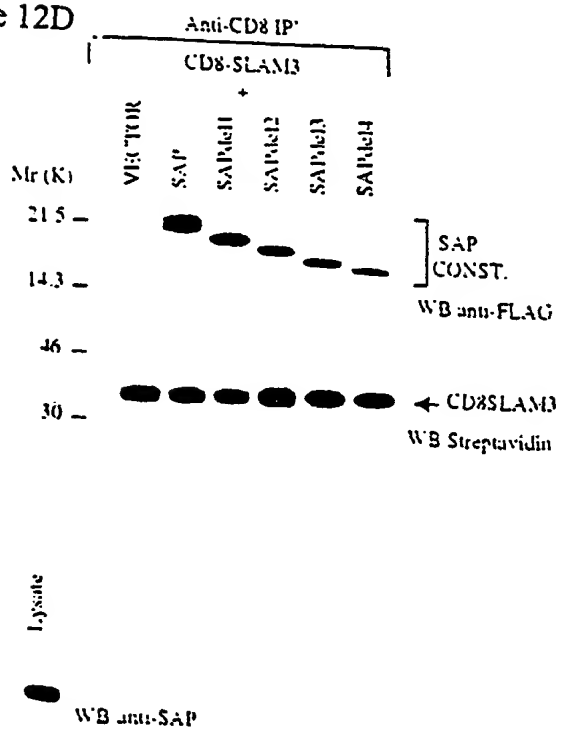
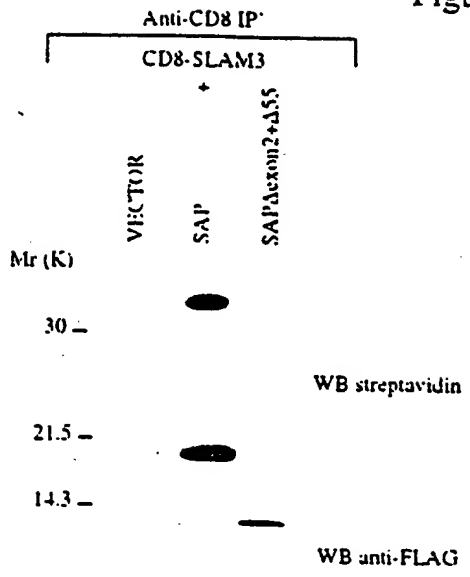


Figure 13A



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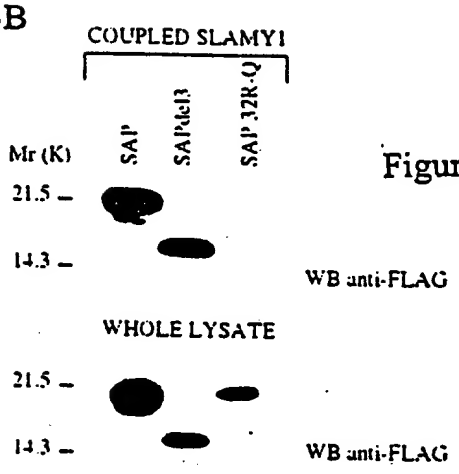


Figure 13C

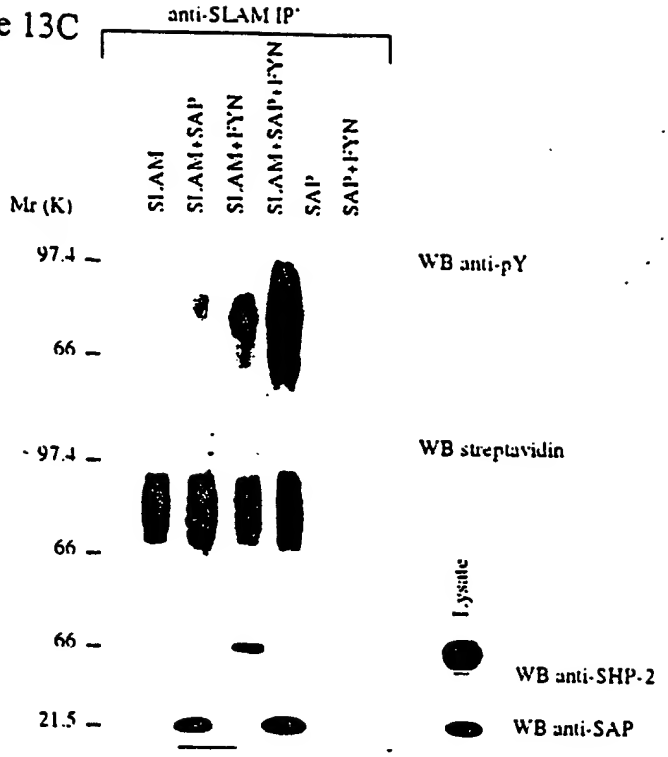
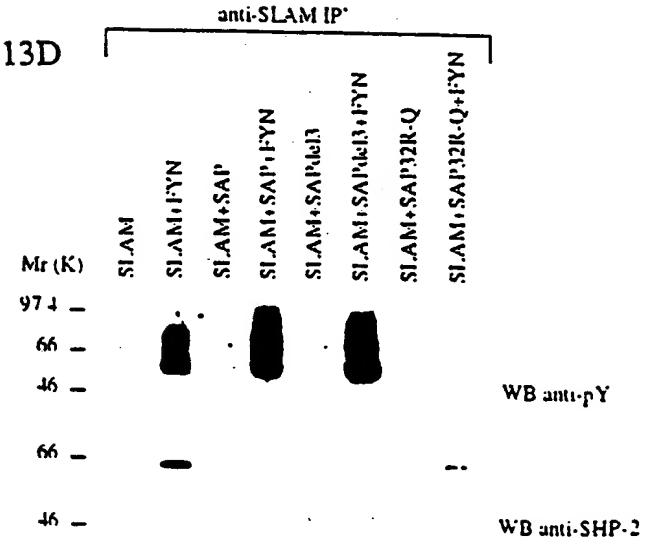


Figure 13D



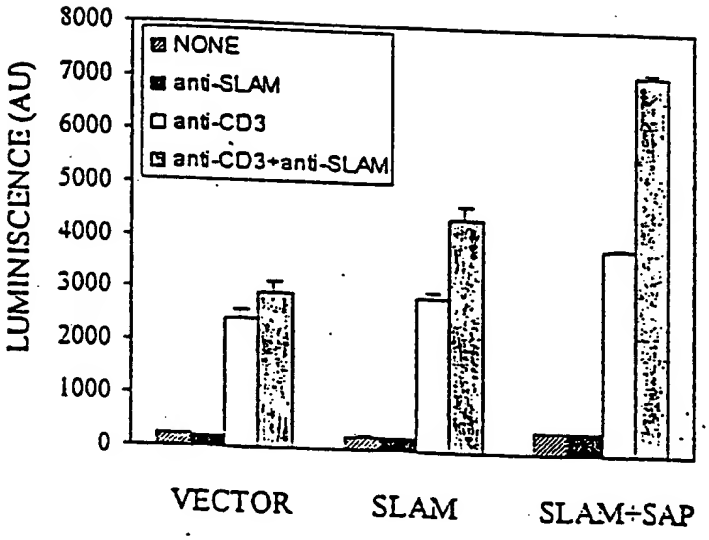

8000
7000
6000
5000
4000
3000
2000
1000
0
LUMINISCENCE (AU)
NONE
anti-SLAM
anti-CD3
anti-CD3+anti-SLAM
VECTOR
SLAM
SLAM+SAP

Figure 14

5' REGION/EXON 1/INTRON 1

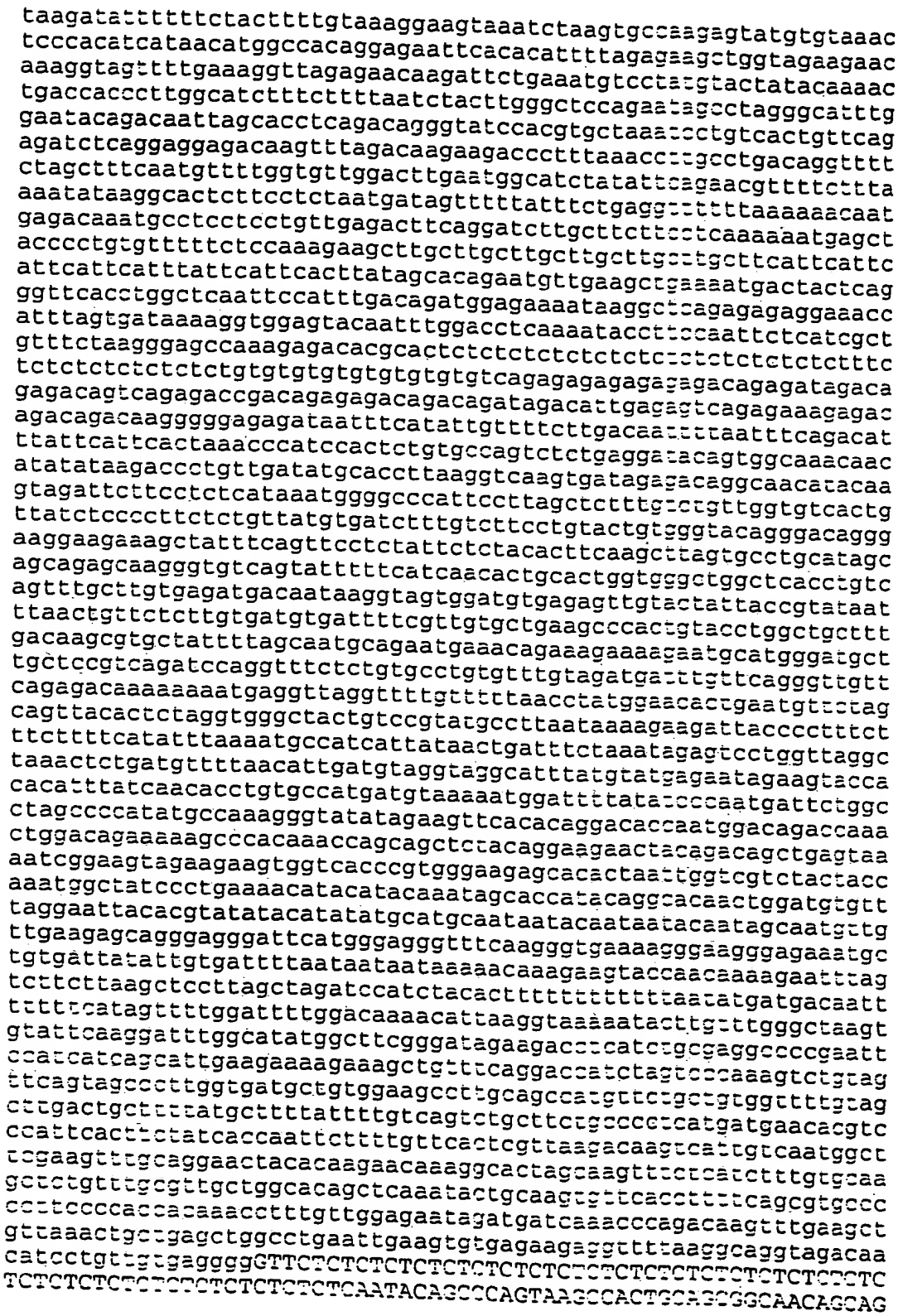


Fig. 15 (sheet 1 of 2)

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tgcagcccagggaaggcagaggtagacagatgtgtgtgagtttcaggtgactaacctgggt
ttacatggcccggtcatccaactaggctacatagataaaaaccaag

Fig. 15 (sheet 2 of 2)

EXON2/INTRON2

GAAACCCTTTGGAGACCAAACAGGTTCTTGGAGTGCCGAggtacagctcttttatttgttt
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Fig. 16

INTRON2/EXON3/INTRON3/EXON4/INTRON4

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Fig. 17 (sheet 1 of 2)

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mSAP Intron/exon sequences

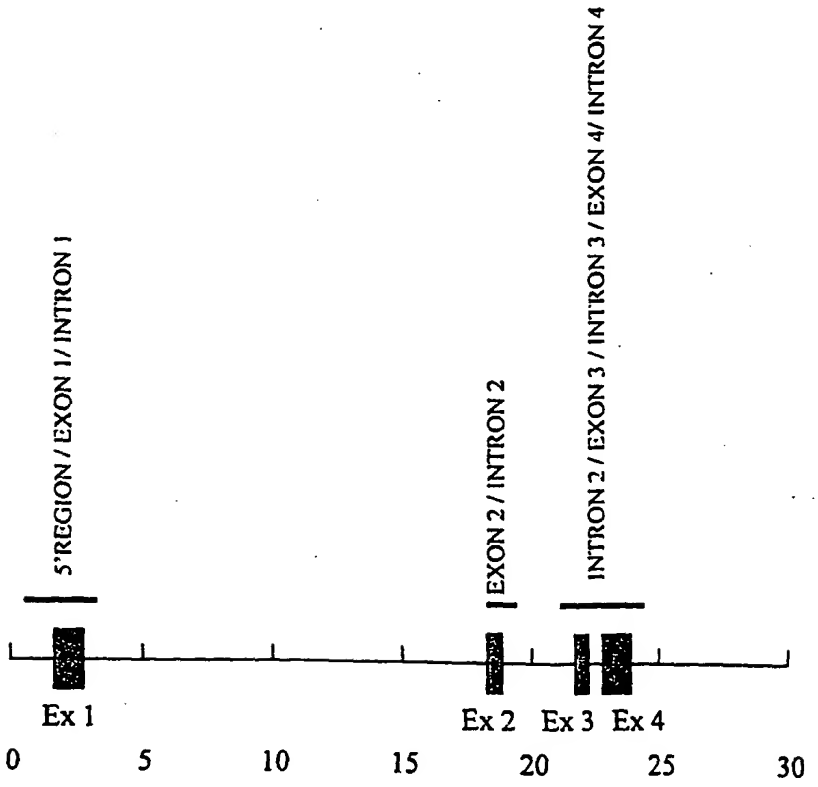
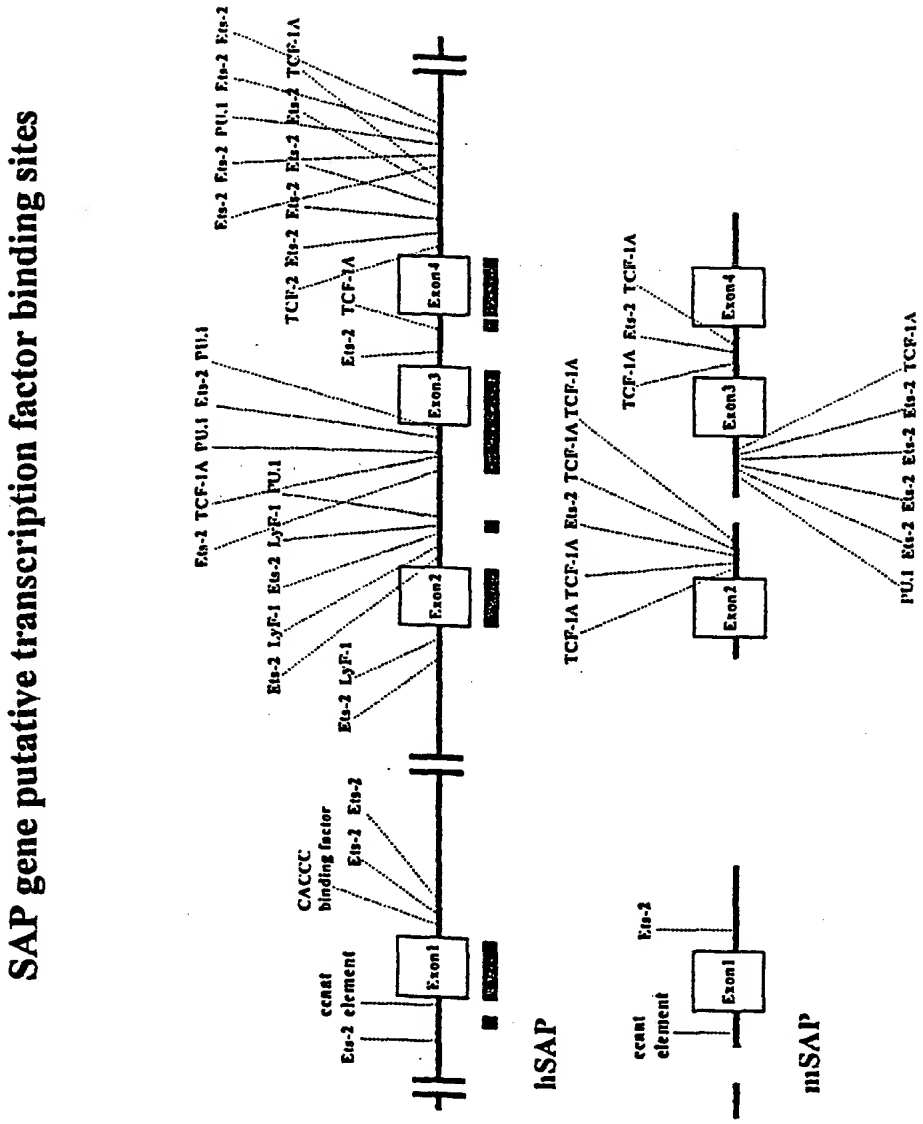


Fig. 18


SAP gene putative transcription factor binding sites
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CACCC binding factor
Ets-2 Ets-2
ccaat element
Ets-2
Exon1
Exon2
Exon3
Exon4
hSAP
TCF-1A TCF-1A Ets-2 TCF-1A TCF-1A
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mSAP
Murine and human SAP genomic sequence homologous region

Fig. 19

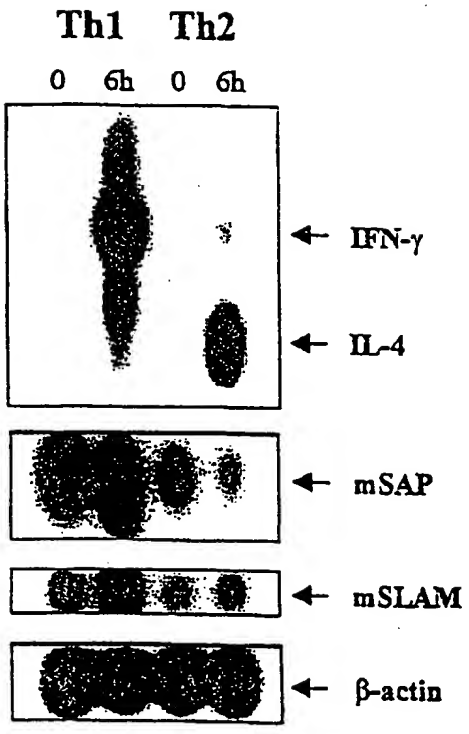

Figure 20

COS CELLS TRANSFECTED

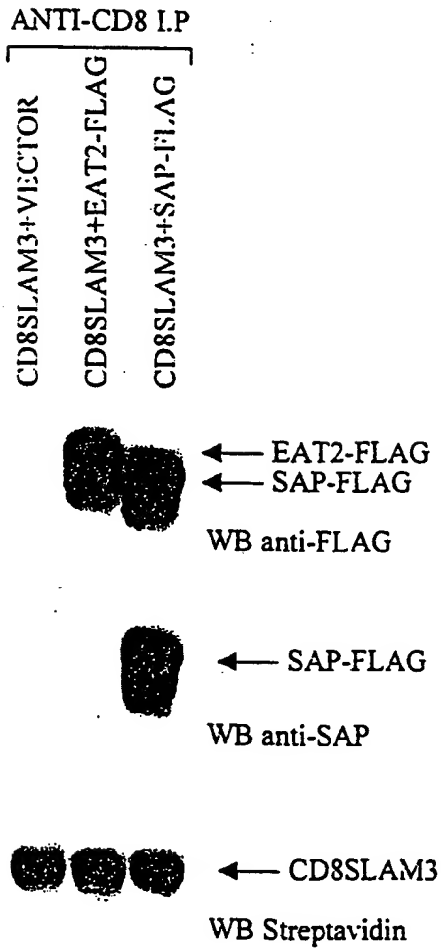

Fig. 21

SEQUENCE LISTING

<110> Beth Israel Deaconess Medical Center

<120> METHODS AND REAGENTS FOR THE UTILIZATION OF SAP FAMILY MEMBER PROTEINS, NOVEL SIGNAL TRANSDUCTION REGULATORS

<130> 01948/061WO2

<140> PCT/US98/xxxxx
<141> 1998-11-19

<150> 08/976,096
<151> 1997-11-21

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35 40 45
Gly Tyr Ile Tyr Thr Tyr Arg Val Ser Gln Thr Glu Thr Gly Ser Trp

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65                  70                  75                  80
Lys Asn Leu Ile Ser Ala Phe Gln Lys Pro Asp Gln Gly Ile Val Ile
                85                  90                  95
Pro Leu Gln Tyr Pro Val Glu Lys Lys Ser Ser Ala Arg Ser Thr Gln
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Asp Ser Glu Ser Val Pro Gly Val Tyr Cys Leu Cys Val Leu Tyr Gln
        35                  40                  45
Gly Tyr Ile Tyr Thr Tyr Arg Val Ser Gln Thr Glu Thr Gly Ser Trp
    50                  55                  60
Ser Ala Glu Thr Ala Pro Gly Val His Lys Arg Phe Phe Arg Lys Val
65                  70                  75                  80
Lys Asn Leu Ile Ser Ala Phe Gln Lys Pro Asp Gln Gly Ile Val Thr
                85                  90                  95
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            100                 105                 110
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35 40 45
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European Patent Office

Application Number
EP 99 92 2568

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☐ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.

☒ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

1-11 and 13-17 (all partially)

☐ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, nam ly:

1. Claims: 1-11 and 13-17 (all partially)

Invention 1
A DNA related to IgA nephropathy comprising a DNA of SEQ. ID: NO: 1 and encoding the protein of SEQ. ID. NO: 34, fragments, mutant and analogs thereof, oligonucleotides derived therefrom, vectors, host cells, methods of producing the protein, antobodies recognizing it, therapeutic and diagnostic compositions,therapeutic uses.

2. Claims: Claims 1-11 and 13-17 (all partially)

Inventions 2-7.
As 1. but respectively referring to SEQ. ID. NO: 2-7 (DNA) and 35-40 (corresponding proteins).

3. Claims: Claims 1-11 and 13-17 (all partially)

Inventions 8-37.
A DNA related to IgA nephropathy comprising one among SEQ. ID: NO: 8-33 and 41-44, fragments and analogs thereof, vectors, host cells, therapeutic and diagnostic compositions,therapeutic uses.

4. Claims: 2-4,10,11,14-17 (all partially)

Invention 38.
An oligonucleotide comprising SEQ. ID. NO: 45 or its complementary, analogs, methods of detection of mRNAs or of inhibiting the expression of a gene using the oligonucleotide, therapeutic and diagnostic compositions, therapeutic uses of the oligonucleotide.

5. Claims: 2-4,10,11,14-17 (all partially)

Inventions 39-99.
As 4. but referring to SEQ. ID. NO: 46-106.

6. Claim : 12

Invention 100.
A mehod of isolating a DNA related to IgA nephropathy form patients' leukocytes.

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 99 92 2568

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

14-02-2002

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0915156 A	12-05-1999	AU 5137498 A	29-06-1998
		EP 0915156 A1	12-05-1999
		CN 1214735 A	21-04-1999
		WO 9824899 A1	11-06-1998
WO 9926980 A	03-06-1999	AU 1599299 A	15-06-1999
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